PATHOBIOLOGICAL STUDIES OF *Vibrio alginolyticus* BIOFILM ON THE DEFENSE FUNCTIONS AND DISEASE RESISTANCE IN GIANT TIGER SHRIMP (*Penaeus monodon*)

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CERTIFICATE

This is to certify that the thesis entitled PATHOBIOLOGICAL STUDIES OF *Vibrio alginolyticus* BIOFILM ON THE DEFENSE FUNCTIONS AND DISEASE RESISTANCE IN GIANT TIGER SHRIMP (*Penaeus monodon*) submitted by Krupesha Sharma, S.R., for the award of degree of DOCTOR OF PHILOSOPHY in VETERINARY PATHOLOGY to the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, is a record of bona-fide research work done by him during the period of his study in this University under my guidance and supervision, and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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January, 2008

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S.R. Krupesha Sharma,

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
cfu	Colony Farming Units
CHO	Carbohydrate
Cu	Copper
d	day
DHC	Differential Haemocyte Count
DO	Dissolved Oxygen
et al	*et alia*
Fig	Figure
FRP	Fibre Reinforced Plastic
g	Gram
h	hour/s
H & E	Haematoxylene and Eosin
IgG	Immunoglobulin G
I/N	Intra Nuclear
LD	Lethal Dose
L-DOPA	L-Dihydroxyphenylalanin
LPS	Lipopolysaccharide
Kg	Kilogram
M	Molar
MAB	Monoclonal Antibody
min	Minute/s
ml	Milli litre
mM	Milli molar
n	Number
N	Normal
NaN_3	Sodium Azide

NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NBT	Nitroblue tetrazolium
O_2	Oxygen
OD	Optical Density
P	Probability
PBS	Phosphate Buffer Saline
PI	Post Infection
PO	Phenoloxidase
proPO	Prophenoloxidase
Rpm	Rotation Per Minute
RPS	Relative Per cent Survival
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrilamide Gel Electrophoresis
SDE	Standard Error
SI	Survival Index
SOD	Superoxide Dismutase
THC	Total Haemocyte Count
TSA	Tryptose Soya Agar
TSB	Tryptose Soya Broth
WSSV	White Spot Syndrome Virus
W/V	Weight by Volume
Zn	Zinc
‰	Parts per thousand
^oC	Degree Celsius
µg	Microgram
µl	Microlitre
µ	Micron

INTRODUCTION

1. INTRODUCTION

Aquaculture is a fast developing industry in India as well as throughout the world with an annual production of over 8 million metric tons. This rapid development is mainly due to its high economic value. It is noteworthy to mention that over half of aquaculture producton is made up of shrimps and prawns. In many countries, the consumption of shrimps has gone up considerably but the natural stocks of these crustaceans have been intensively depleted. Alternatively, shrimp culture has been adopted and consolidated as one of the largest profitable aquaculture activities all over the world. World production of farmed shrimp has substantially increased during the last 20 years, mainly due to an increase in farming area and higher stocking densities. Asia has always led world production of cultured shrimp with a substantial market value.

Tiger shrimp, *Penaeus monodon* which is a euryhaline, is naturally distributed from the east coast of Africa, Red Sea to Pakistan, Malaysia, Philippines and Australia. *Penaeus monodon* is commercially important in several pacific countries and is the most widely cultured species in Asia except in China and Japan, where the main crops are *Penaeus chinensis* and *Penaeus .japonicus*, respectively.

Both partial and full cultures carry risk of monetary losses due to disease outbreaks, mortality and meat quality. The environmental contamination due to farm discharges into estuaries and bays and the stress induced in ponds by high stocking densities are the factors that predispose the shrimps to several diseases.

The shrimp industry has often been plagued by many infectious diseases (Lightner *et al.*, 1983; Kroll *et al.*, 1991; Hasson *et al.*, 1995) causing great loss in production. The sustainability of shrimp industry

depends largely on disease control programmes and health status of shrimp. Strategies to counter infectious diseases cannot be successful without the knowledge of shrimp immunity which will help to unearth the mechanisms of pathogen evasion of the host responses. From this point of view, the immune system of shrimp serves as a tool to assess the health status. Bachere *et al.* (1995) have emphasized the value of immune parameters as biomarkers in ecotoxicology. Invertebrates do not possess an acquired immunity equivalent to vertebrates; instead they have an innate immune system. This includes melanization by activation of prophenoloxidase activating system, clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action and cell agglutination. Research in penaeid shrimp immunology has recently gained priority due to increased impact of diseases on sustainability and economic durability of shrimp aquaculture (Munoz *et al.*, 2002).

In order to reduce the impact of diseases, shrimp industry has been using antibiotoics, immunostimulants, and diverge type of feed additives. Although bacterial infections are controlled to some extent by antibiotic treatment, use of antibiotics has lead to environmental hazards and development of antibiotic-resistant pathogens. Further, viral diseases limit the efficacy of antibiotic treatment. The practice of use of antibiotics in shrimp culture has been phased out in many countries (Smith *et al.*, 2003). Selective breeding programmes and use of genetically modified strains are still a long way from providing an ethically acceptable and commercially viable means of reducing the problem posed by diseases. Therefore, there has been a growing interest in finding ways to protect shrimps prophylactically in a manner conceptually equivalent to the use of vaccines in livestock, poultry and farmed fish.

Research conducted during the last 15 years on enhancement of immunity and disease control in shrimp has focused enough light on a range of compounds. Immunostimulants receiving most attention and claims for success in promoting survival of shrimp against exposure to infectious microorganisms comprise five main types: viz., live bacteria, killed bacteria, glucans, peptidoglycans and lipopolysaccharides (Smith *et al.*, 2003).

Wherever live bacteria were used, the strains of interest often included *Vibrio* spp. or other mild pathogens. Killed bacteria (bacterins) are frequently obtained from either freeze-dried, heat killed or formalised strains of known pathogens. Both live bacteria and bacterins appear to mimic non-virulent or attenuated pathogens similar to vaccines in higher vertebrates. Peptidoglycan, glucan and LPS stimulants are often derived from cell walls of non-pathogenic bacteria or fungi.

Biofilm glycocalyx which embeds the cells in a growing biofilm community, renders immense protective resistance from the action of surfactants (Govan, 1975), antibiotics (Hoyle *et al.*, 1990), antibodies (Baltimore and Mitchell, 1980) and phagocytic cells (Anwar *et al.*, 1992). Considering the resistant nature of biofilm and inconsistent responses to oral vaccines in fish due to antigen destruction in the gut, the concept of using bacterial biofilm for oral vaccination has been hypothesized and validated (Azad *et al.*, 1999). The better performance of biofilm vaccine in terms of increased immune response and protection in fish was attributed to superior antigen delivery to the lymphoid tissues as demonstrated by antigen localization using monoclonal antibodies (Azad *et al.*, 2000).

Several published research reports on products with immunostimulatory properties in shrimp are available (Smith *et al*, 2003). However, there is a paucity of information on the use of bacterial

biofilm in enhancing the immune potential of shrimp. Hence, it was considered worthwhile to make a systematic study on the effects of biofilm cells of *Vibrio alginolyticus*, a most common opportunistic pathogen of shrimp, on the defense functions and disease resistance in *Penaeus monodon*, with the following objectives:

1. To study the defense functions of *Penaeus monodon* exposed to biofilm and free cells of *Vibrio alginolyticus*
    a. To standardize *in vitro* development of biofilm cells of *Vibrio alginolyticus* and study its growth kinetics
    b. To standardize dose requirement of biofilm cells of *Vibrio alginolyticus* to enhance the immune potential of *Penaeus monodon*
2. To characterize the pathomorphological changes caused by pathogenic *Vibrio alginolyticus* and white spot syndrome virus in *Penaeus monodon* exposed to biofilm and free cells of *Vibrio alginolyticus*
    a. To compare the effects of biofilm and free cells of *Vibrio alginolyticus* on the resistance of *Penaeus monodon* to pathogenic *Vibrio alginolyticus*
    b. To compare the effects of biofilm and free cells of *Vibrio alginolyticus* on the resistance of *Penaeus monodon* to white spot syndrome virus

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Prelude to review

Production from shrimp industry is seriously affected by environmental degradation and infectious and non-infectious diseases. Since vaccination or treatment with antibiotics is not a feasible proposition in shrimp culture, interest is being focused on developing compounds that confer protection and/or enhance immune reactivity to likely pathogens in shrimp. These compounds are thought to act as immunostimulants because of their known effects on the crustacean immune system *in vitro* (Smith *et al.*, 2003).

For convenience, the literature pertaining to the present study has been reviewed under the following heads:

- Immune system and immune response in shrimp
- Shrimp diseases with reference to vibriosis and white spot syndrome
- Immunomodulators of shrimp immune system
    - Research on immunomodulators in shrimp/ prawn other than *Penaeus monodon*
    - Research on immunomodulators in *Penaeus monodon*
    - Environmental factors acting as immunomodulators of shrimp immune system
    - Bacterial biofilm as immunomodulator in aquaculture

2.2 Immune system and immune response in shrimp

An essential component of immunity is the mechanism of surveillance by which an organism can detect the presence of "non-self"

molecules. A good non-self recognition system should also stimulate defensive responses, including those mediated by cells. In vertebrates, the immune defense includes adaptive memory, specific immunoglobulins and specialized cells as well as non-specific responses through phagocytic cells and natural killer cells.

Invertebrates do not have antibodies, albeit they possess proteins with domains belonging to the immunoglobulin super family (Lanz Mendoza and Faye, 1996) by which they are able to recognize and destroy invading microorganisms or parasites. Proteins involved in the recognition of cell wall components from microorganisms such as lipopolysaccharide (LPH) and β-1, 3- glucans have been found in invertebrates. However, these proteins are unable to destroy foreign matters and a phagocytic activity is required (Ratcliffe *et al.,* 1985).

Often while describing the components of immune response in crustaceans, a division into humoral and cellular components is used. The humoral factors comprise molecules that act in the defense without direct involvement of cells although many of these factors are originally synthesized and stored in the blood cells. Consequently, the actions with the direct participation of blood cells are understood by the term cellular response (Holmblad and Soderhall, 1999). For evaluation of these humoral and cellular parameters of immune response in shrimp, simplified procedures have been developed (Rodriguez and Le Moullac, 2000). Several workers have been studying the quantification of different cellular and humoral parameters of the immune response in shrimp (Bachere *et al.*, 1995; Rodriguez *et al.*, 1995; Sung *et al.*, 1996; Vargas – Albores *et al.*, 1996).

2.2.1 Shrimp blood cells

Crustaceans have an open circulatory system with absence of vertebrate red blood cells but analogues of the white blood cells which perform the functions of both exist. In invertebrates, the circulating cells are called as haemocytes which are essential in immunity, performing functions such as phagocytosis, encapsulation and lysis of foreign cells (Smith and Soderhall, 1983; Ratcliffe *et al.*, 1985; Soderhall and Smith, 1986; Johansson and Soderhall, 1989).

2.2.1.1 Types of haemocytes

Crustaceans have three morphologically different haemocyte types: hyaline, semi granular and granular cells (Bauchau, 1980). Granular cells have a large number of secretory granules containing components of prophenoloxidase (proPO) system. Semi-granular cells appear to be the most sensitive ones and react first during an immune response by degranulation. Release of vesicle contents can stimulate the granular cells to degranulate as well (Rodriguez and Le Moullac, 2000).

2.2.1.2. Functions of haemocytes

Functions of haemocytes based on isolated population of cells by different workers are presented as here under:

Haemocyte type	**Functions in immunity**
Hyaline cells	Phagocytosis[1]
Semi granular cells	Encapsulation[2]
	Phagocytosis (limited) [1]
	Storage and release of the proPO system[3]
	Cytotoxicity[4]
Granular cells	Storage and release of the proPO system[3]
	Cytotoxicity[4]

[1] Smith and Soderhall (1983)

[2] Persson *et al.* (1987)

[3] Johansson and Soderhall (1985)

[4] Soderhall *et al.* (1985)

2.2.1.3 Identification of different types of haemocytes

2.2.1.3.1 Cytochemical techniques

Hose *et al.* (1987) reported that the acid phosphatase activity was more abundant in semi-granular cells while hyaline cells were distinctively stained by Sudan Black. Sequeira *et al.* (1996) performed cytochemical staining of haemocyte sub-populations separated by flow cytometry and reported positive peroxidase activity only in granular cells.

2.2.1.3.2 Molecular techniques

An alternative method for cell identification is the use of monoclonal antibodies (MAbs) to find out antigenic markers of different cell types. Using MAbs against different sub-populations of haemocytes separated in a percoll gradient, it was found that hyaline cells share epitopes with semi-granular cells and that an antigen was specifically expressed on semi-granular cells in *Penaeus japonicus* (Rodriguez *et al.*, 1995).

2.2.2 Clotting and wound healing

Haemolymph coagulation is an essential defense response in crustaceans that prevents loss of haemolymph through breaks in the exoskeleton and dissemination of bacteria throughout the body (Martin *et al.*, 1991). Coagulation is a rapid and powerful process in crustaceans. Clotting is best described in *Limulidae*, horseshoe crab, where a cascade of proteinases leads to activation of a clotting protein, coagulogen (Kawabata *et al.*, 1996). By the action of transglutaminase

which is stored in the haemocytes and released on activation, covalent cross-links are created so that a clot is formed. Montano-Perez *et al.* (1999) purified the clotting protein of white shrimp *Penaeus vannamei* by affinity chromatography in a heparin–agarose column. The protein named clotting protein was found to be a lipoglycoprotein composed of two 210-kDa subunits covalently bound by disulfide bridges.

2.2.3 Antimicrobial peptides

Antimicrobial peptides and proteins have been well studied in arthropods (Hetru *et al.*, 1994; Iwanaga *et al.*, 1998), where families of antimicrobial molecules have been isolated and characterized. While ample literature is available on different antimicrobial peptides in crab, research in shrimp is scarce. Destoumieux *et al.* (1997) fully characterized the three members of new family of antimicrobial peptides in penaeid shrimp. These peptides, named penaeidins, are the first antimicrobial molecules to be discovered in penaeid shrimp. The penaeidins are 5.5 to 6.6 kDa peptides which combine a proline-rich amino-terminal domain and a carboxyl-domain containing six cysteines engaged in three disulfide bridges. The anti bacterial activity of these penaeidins compared with other effectors of the innate immunity has been extensively reviewed by Bachere *et al.* (2000).

Chiou *et al.* (2007) studied the expression and characterization of *Penaeus monodon* penaeidin in various tissues during early embryonic development and moulting stages using polymerase chain reaction by specific primers. They observed that mo-penaeidin gene consisted of 1348 bp containing one intron (680 bp) and two exons (210 and 458 bp) with an open reading frame of 222 bp which encodes a protein of 74 amino acids including a signal peptide of 19 amino acids. The mo-penaeidin mRNA was detected in various tissues including ovary and mandibular organ. The penaeidin mRNA was found to be present in one

cell to post larva stage with higher level at nauplius I. Also, its expression was significantly higher during intermoult stage.

2.2.4 Phenoloxidase system and melanin formation

The prophenoloxidase (proPO) activating system is one of the best studied immune system in crustaceans with numerous published works on crayfish. The phenoloxidase (PO) is responsible for the melanization process in arthropods where melanin synthesis is involved in the process of sclerotization and wound healing of the cuticle as well as in defense reactions (nodule formation and encapsulation) against invading microorganisms entering the hemocoel (Soderhall, 1982; Ratcliffe *et al.*, 1985; Sugumaran, 1996). The PO enzyme results from the activation of proPO enzyme which is present as an inactive zymogen in haemolymph or cuticle. PO is a bifunctional copper containing enzyme which catalyses *o*-hydroxylation of monophenols and the oxidation of phenols to quinines (Sugumaran, 1996). Thus, the enzyme is able to convert tyrosine to DOPA, as well as, DOPA to DOPAquinone followed by several intermediate steps that lead to the synthesis of melanin, a brown pigment (Sritunyalucksana and Soderhall, 2000).

The proPO system can be activated by an endogenous activating system and exogenous agents such as lipids, detergents, organic solvents, and microbial elicitors like β-1, 3-glucan, lipopolysaccharide, and peptidoglycan (Ashida and Soderhall, 1984; Ashida and Yamazaki, 1990). In crustaceans, proPO has been demonstrated to be confined to haemocyte granules (Barrett, 1987) and it could also be activated by different chemical and microbial elicitors (Brivio *et al.*, 1992). In addition, Ca^{2+} is required for the conversion of the proPO-activating enzyme to an active proteinase that transforms proPO to active phenoloxidase.

Biochemical studies on the shrimp proPO system have been carried out in *Farfantepenaeus californiensis* (Johansson and Soderhall, 1985; Brivio *et al.*, 1992; Burks and Fuchs, 1995), *Farfantepenaeus paulensis* (Johansson and Soderhall, 1992) and *Penaeus monodon* (Lanz Mendoza *et al.*, 1993). proPO has been purified and characterized from haemocytes of *Pacifastacus leniusculus* (Leonard *et al.*, 1985) and *F. californiensis* (Burks and Fuchs, 1995) and the molecular masses were 76 and 114 kDa, respectively. *Penaeus monodon* proPO gene was purified and cloned by Sritunyalucksana *et al.* (1999a). The authors reported that shrimp proPO had a 3002 bp cDNA and contained an open reading frame of 2121 bp encoding a putative polypeptide with 688 amino acids and a molecular mass of 78.7 kDa.

2.2.5 Pattern recognition proteins

The first immune process in crustaceans is the recognition of invading microorganism which is mediated by the haemocytes and plasmatic proteins (Vargas-Albores *et al.*, 1996). Crustaceans do recognize common characteristics present in bacteria and fungus such as lipopolysachharides and β-glucans. There is little information about the molecular mechanisms that mediate recognition; however, in crustaceans, several types of modulator proteins have been described that recognize cell wall components of microorganisms (Bachere, 2000).

Although most research on identification of pattern recognition proteins is focused on crayfish, scanty literature is available with regard to shrimp. In shrimp, both LPS (Vargas-Albores *et al.*, 1993; Maheswari *et al.*, 1997) and β-glucan (Vargas-Albores *et al.*, 1996) binding proteins are present as possible recognition proteins. Vargas-Albores and Yepiz-Plascencia (2000) reported that the mechanism of action of invertebrate recognition protein appeared similar to vertebrate antibodies where, after

reaction with an antigen, the immunoglobulin can activate cellular functions (degranulation and phagocytosis) or plasma complement.

2.2.5.1 β-1, 3 glucan binding protein

β-glucan binding protein (BGBP) and its role in shrimp immune response have been reviewed by Vargas-Albores and Yepiz-Plascencia (2000). Other than two insect species and fresh water crayfish, this protein has been purified in yellow leg shrimp, *P. californiensis* (Vargas-Albores *et al.*, 1996) and white shrimp *P. vannamei* (Vargas-Albores *et al.*, 1997). This protein appeared to be widely distributed among the crustaceans conserving most of its antigenic properties, since a monospecific polyclonal antiserum against *P. leniusculus* BGBP could recognize BGBP from different crustaceans including several shrimp species. In addition, antibodies prepared against purified yellow leg shrimp BGBP clearly detected a 100 kDa protein in plasma from *P. Vannamei* and *P. Stylirostris* (Vargas-Albores *et al.*, 1996).

It was found that BGBP was unable to induce release and activation of the proPO system, but the protein-glucan complex was able to react with the circulating cells and increase the effect of glucans on the proPO system (Borracco *et al.*, 1991; Johanssan and Soderhall, 1992; Vargas-Albores, 1995). Thus the recognition proteins are capable of activating cellular activities only after reacting with the microbial carbohydrate (LPS, peptidoglycon and glucans).

2.2.5.2 Lectins

Occurrence, specificity and biological role of crustacean lectins, primarily those of shrimps have been reviewed by Marques and Borracco (1999). Earlier studies have emphasized the possible role of lectins as non-self-recognition molecules in vertebrate and invertebrate immunity (Renwrantz, 1986; Arason, 1996; Matsushita, 1996; Vasta *et al.*, 1999;

Wilson *et al.*, 1999). Due to the fact that lectins have the ability to bind carbohydrate and promote the agglutination of different cells such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be having a potential role in invertebrate non-self-recognition reactions.

In the penaeid shrimp, *P. monodon*, Ratanapo and Chulavatnatol (1992) reported agglutination of highly pathogenic *Vibrio vulnificus* by a purified lectin called monodin. In the other penaeid, *Penaeus californiensis*, Vargas-Albores *et al.* (1993) investigated the ability of purified lectin to react with different marine species of *Vibrio*. They demonstrated that the agglutinin of this penaeid was able to react with at least three different *Vibrio* species, *V. vulnificus*, *V. fischeri and V. parahaemolyticus*. This reaction was specific and the agglutination of *V. parahaemolyticus* could be inhibited by LPS which suggested that this natural ligand of the penaeid lectin could be one effective sign that trigger the shrimp immune system. In the prawn *P. longirostris*, Fragkiadakis and Stratakis (1995) also reported that purified lectins from the haemolymph that recognised *N*-acyl aminosugars strongly agglutinated formalin-fixed *Pseudomonas aeruginosa* and *E. coli*. The observations of Vazquez *et al.* (1993, 1996, 1997) on the lectins of the haemolymph of the freshwater prawn *Macrobrachium rosenbergii* are of particular interest. The authors purified and characterized a lectin from the prawn haemolymph and showed that it had the ability to agglutinate several bacteria by recognizing *O*-keto and *O*-methyl containing sugars and *N*-acetyl-sugar in the cell wall. In a later report, Vazquez *et al.* (1997) demonstrated that the granulocytes of *M.rosenbergii*, in spite of expressing a surface receptor which seemed to correspond to the humoral purified lectin, had the ability to recognize foreign cells in an apparently non-mediated sugar recognition basis.

Ratanapo and Chulavatnatol (1992) reported an elevation of the lectin monodin level in most of *P.monodon* suffering from *V. vulnificus* infection. On the other hand, in the same species, Sritunyalucksana *et al.* (1999b) failed to induce increase in lectin concentration *in vitro* and *in vivo* by using components of microorganism cell wall such as LPS, β-glucans, peptidoglycan and also commercial stimulants.

2.2.6 Phagocytosis

Phagocytosis, a most common reaction of cellular defense involves internalization of particles or microorganisms into the cell which later form a digestive vacuole called phagosome. The elimination of phagocytosed particles involves the release of digestive enzymes into the phagosome and generation of reactive oxygen intermediates (ROIs), known as respiratory burst. The first ROI generated during this process is the superoxide anion (O_2^-). Subsequent reactions will produce other ROIs such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$). Hydrogen peroxide can be converted to hypochlorous acid (HOCL-) via the myeloperoxidase (MPO) - H_2O_2-CL system, forming a potent antibacterial system (Bayne, 1990).

In penaeid shrimp, most studies on phagocytosis have been performed through observations of clearance of injected bacteria or particulate materials (Martin *et al.*, 1993). Most studies on ROIs generation in invertebrates have been conducted in mollusks (Bachere *et al.*, 1991; Pipe, 1992; Anderson, 1994). Quantitative procedures have been applied for shrimp ROIs generation such as nitroblue tetrazolium (NBT) reduction technique for the measurement of intracellular O_2^- and the reduction of ferricytochrome C for extracellular O_2^- (Rodriguez and Le Moullac, 2000).

Song and Hsieh (1994) described for the first time the oxidative metabolism in *P.monodon*. They measured O_2^- using NBT reduction technique and H_2O_2 by HRP dependent oxidation of phenol red. Bachere *et al.* (1995) demonstrated the existence of respiratory burst in *P. japonicus* induced by zymosan. Le Moullac and Haffner (2000) emphasized the importance of respiratory burst in *P. vannamei* and its value as biomarker of environmental disturbances.

Deachamag *et al.* (2007) studied the expression of a phagocytosis activating protein (PAP) gene in immunized *P.monodon*. It was reported that immunostimulation with inactivated *Vibrio harveyi* induced the PAP gene which is a ribosomal protein L26 (RPL26) gene and facilitated the protective defense against WSSV infection. The expression level of the PAP gene served as an indicator of the immune response in cultured shrimp.

2.2.7 Plasma protein

One of the important functions of haemolymph in crustaceans is to transport molecules such as the respiratory protein (haemocyanin) which is the most abundant molecule of the haemolymph (60 to 95 per cent of total protein) followed by the clotting protein and other humoral components (Djangmah, 1970). In shrimp, concentration of plasma proteins is related to moult cycle. Chen and Cheng (1993) observed that in *P. japonicus*, the plasma protein levels were lower during post moult as opposed to higher levels found in early pre-moult.

2.3 Shrimp diseases with reference to vibriosis and white spot syndrome

Among the diseases of shrimp, the diseases caused by viral, bacterial, fungal, protozoan and rickettsial etiologies have gained considerable importance (Lightner, 1988; Brock and Lightner, 1990).

During the last decade, it has been reported that infectious diseases caused by virus followed by bacteria have caused massive mortalities in shrimp culture around the globe (Flegel, 2006).

2.3.1 Vibriosis

Vibrio spp. is a Gram negative, oxidase positive and motile organism. Various species of *Vibrio* like *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. dansela, V. harveyi, V. anguillarium, V. nereis and V. fluvialis* have been described as the principal pathogenic species that affect penaeid shrimp (Lightner *et al.*, 1993). This bacterium is known to take advantage of ecological changes in culture system and to cause periodic diseases in shrimp (Skjermo and Vadstein, 1999). The effect and severity of disease in shrimp are mainly related to the type of *Vibrio* spp., level of infection, water quality, feed and shrimp quality at the time of stocking into pond (Lightner *et al.* 1983). Yasuda and Kitao, (1983) observed low growth rate of shrimp larvae at protozoeal stage when *Vibrio* were present at higher concentration (10^7 cfu/g) in water and shrimp gut. Mortalities in *P. monodon* and *P. merguiensis* larvae have been observed in Indonesia, Thailand, Philippines and other countries (Johnson, 1994). The mortality reported ranged from insignificant to 100 per cent, particularly in post larvae and juvenile shrimps. In juvenile and adult shrimps, diseases due to *Vibrio* are commonly known as Sea gull syndrome (Lightner, 1983), Red disease syndrome (Alapide-Tendencia and Dureza, 1997), Tea brown gill syndrome (Ruangpan *et al.*, 1999) and Syndrome-93 (Costa *et al.*, 1998). The species primarily involved were *V. harveyi, V. fluvialis, V. parahaemolyticus and V. penaeicida.* In larvae and postlarvae, vibriosis is classified as oral/enteric vibriosis and appendage/cuticular vibriosis.

2.3.2 White spot syndrome

White spot syndrome is a disease caused by white spot syndrome virus (WSSV) in shrimps (Lo *et al.*, 1997). WSSV infection is characterized by gross lesions of white spots of various sizes embedded in the cuticle at the later stages of infection. These lesions were first reported from an outbreak that occurred in *P. japonicus* in Japan in 1993. The causative agent was a new bacilliform virus which is now called white spot syndrome virus by general consensus (Lightner and Redman, 1998). White spot syndrome virus was originally called as baculovirus based on its cylindrical morphology and histological lesions that resembled "non-occluded" baculoviruses (Wongteerasupaya *et al.*, 1995). It is now known that WSSV is a tailed, rod shaped, double stranded DNA virus with a very large circular genome in the order of 300 kbp. Since the genome had no significant homology to any known virus (Yang *et al.*, 2001), a new viral family (Nimaviridae) and genus (Whispovirus) were created to accommodate it (Mayo, 2002).

2.4 Immunomodulators of shrimp immune system

Immunostimulants, in general increase resistance to infectious diseases by enhancing non-specific defense mechanisms. Since there is no memory component involved, the response is likely to be of short duration (Sakai, 1999).

The disease out breaks pose a continual threat to the existence of any shellfish farm or hatchery. Once an infection occurs it can prove devastating to the entire stock. To some extent good husbandry practices may help but additional forms of protection are necessary to prevent epidemics. Application of antibiotics or other chemicals is undesirable due to heavy cost involved and also risk of contamination of both environment and final product (Grant and Briggs, 1998). Repeated application of antibiotics in the long term may lead to spread of drug

resistant pathogens (Smith *et al.*, 1994). Hence, there is a need to maximize the immunocompetence of the stock while minimizing the use of therapeutic agents (Bachere *et al.*, 1995). As of now, there is no evidence that crustaceans share with vertebrates, clonally derived subsets of cells that permit specific, adaptive and 'memory-based' immunity that is the basis for conventional vaccination regimen. Further, crustaceans do not appear to possess immunoglobulin molecules and a complete complement system or there is nothing to suggest that they demonstrate the rearrangement of genes that underpin the generation of diversity within the vertebrate immune system. Hence, immunostimulants must act on the innate immune system of crustaceans and therefore, it can be presumed that these immunostimulants can boost the non-specific defense system to improve surveillance and reaction towards potential non-self threats (Smith *et al.*, 2003).

2.4.1 Research on immunomodulators in shrimp or prawn other than *P.monodon*

To examine the potency of oral administration of peptidoglycan (PG) derived from *Bifidobacterium thermophilum*, Itami *et al.* (1998) administered PG to kuruma shrimp (*P. japonicus*) through diet at 0.2 mg/kg body weight/day for 7 consecutive days, alternated with 7 days without PG throughout a 95-day test period. After sampling the shrimp on Day 65 and 95, they were challenged with *Vibrio penaecida* and WSSV individually. The survival rate of PG-fed group was significantly higher than the control in both the challenge studies. Further, Phagocytic index of PG-fed shrimps was higher than that of the control.

Enhancement of resistance against vibriosis in juvenile *P.vannamei* by supplementation of diets with different yeast products was evaluated by Scholz *et al.* (1999). The shrimps were reared on five different

experimental diets containing *Saccharomyces cerevisiae* (1 per cent), β-glucan extracted from *S. cerevisiae* (0.1per cent), *Phaffia rhodozyma* (1 per cent), experimental yeast HPPR1 (1 per cent) and a control diet. Twenty-four hours after immersing the shrimps in a viable cell suspension of *V. harveyi*, the shrimps which were fed with *S. cerevisiae*, *P. rhodozyma*, and HPPR1 and control diet had effectively cleared the bacteria from the haemolymph while the shrimps fed with glucan diet showed elevated bacterial count. Determination of phenoloxidase activity of shrimps showed a significant difference among the five treatments with phenoloxidase activity for the *Phaffia*-treated shrimps being significantly lower than any other diets except the β -glucan diet.

Takahashi *et al.* (2000) studied the enhancement of disease resistance against Penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of *P. japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS) and observed that the oral administration of LPS increased the phagocytic and PO activity of shrimp haemocytes. Also, virus-inactivating activity was induced in the haemolymph which might play an important role in controlling the viral infection in shrimp.

The immunomodulatory action of superoxide dismutase (SOD) and its possible use as an indicator of immune response in American white shrimp (*Litopenaeus vannamei*) was studied by Campa-Courdova *et al.* (2002a). The SOD activity in haemocytes was quantified to evaluate whether β-glucan and sulfated polysaccharide induced immunostimulatory activity. The haemocytes showed increased levels of SOD activity and decreased total haemocyte count within 24 h after administration of immunostimulants. The total haemocyte count and total soluble haemolymph protein increased over normal values after 48–120 h. It was concluded that the single immunostimulation with β-

glucan and sulfated polysaccharide was sufficient to generate an increase in the antioxidant activity of *L. vannamei* SOD.

Lopez *et al.* (2003) designed a study to determine the effect of dietary β 1-3 glucan (BG) and a mega dose of vitamin C on the immunological system in *L. vannamei* juveniles. The authors recorded higher blood protein, total blood cells, granular cells and PO activity in shrimp fed with vitamin C as compared to the remaining treatments.

Pascual *et al.* (2004a) fed shrimps with a high (HCHO: 44 per cent) or a low (LCHO: 3 per cent) carbohydrate diet for 55 d to *L. vannamei* juveniles. The authors found a direct relation between dietary CHO and lactate, protein and haemocyte levels indicating that dietary CHO was used for protein synthesis via transamination pathways in wild shrimp and in farmed shrimp these parameters were inversely proportional to dietary CHO level indicating that the capacity to synthesize protein from dietary CHO was repressed in cultured shrimp.

In a study designed to evaluate the effect of dietary protein level on survival and immunological condition of *L. vannamei* juveniles, Pascual *et al.* (2004b) observed not only a reduction of haemocytes in shrimp fed sub-optimal dietary protein levels but also reduction in zymogens contained in haemocytes, i.e., prophenoloxidase (ProPO) system, peneidins and their activities (phagocytosis, coagulation).

In an experiment to study the effect of replacement of fish meal by meat and bone meal and poultry by-product meal in diets on the growth and immune response of *Macrobrachium nipponense*, Yang *et al.* (2004) observed no significant difference in immunological parameters including total haemocyte count, PO activity and respiratory burst while the values for all the immunological parameters studied in the control group were significantly higher than those in replacement group.

Maggioni *et al.* (2004), in a study to examine the modulation of some hemato-immunological parameters in female *L. vannamei* submitted to unilateral eyestalk ablation and whose diet was supplemented with high doses of vitamin C as a form of immunostimulation, observed absence of significant changes in the hemato-immunological parameters suggesting the existence of a compensatory mechanism induced by the non-ablated eyestalk.

Cheng *et al.* (2005b) observed that the total haemocyte count, PO activity, respiratory burst and phagocytic activity and clearance efficacy of the shrimp *L. vannamei* increased significantly when shrimps were administered sodium alginate at different concentrations with feed for five months. The survival of the shrimp after challenging them with *V. alginolyticus* was also significantly higher in sodium alginate fed group.

Protective effect of chitin and chitosan against *V.alginolyticus* in white shrimp, *L. vannamei* was studied by Wang and Chen (2005), after injecting the shrimps with either chitin or chitosan at different concentrations. It was observed that the survival of shrimps that received chitin or chitosan was significantly higher than that of control shrimp at the termination of the experiment. Also, it was found that shrimp which received chitin at 6 µg/g or chitosan at 2 and 4 µg/g had higher total haemocyte count, respiratory burst, PO activity and phagocytic activity against *V. alginolyticus* indicating that chitin and chitosan increased the immune ability and resistance to *V. alginolyticus* infection in *L. vannamei.*

The immunostimulatory effects of hot water extracts of *Gracilaria tenuistipitata* (Hou and Chen, 2005) and *Gelidium amanasii* (Fu *et al.*, 2006) on the white shrimp *L.vannamei* and their resistance against *V. alginolyticus* were investigated. In these studies, total haemocyte count, PO activity, respiratory burst, phagocytic activity and clearance efficacy

to *V.alginolyticus* were examined after shrimps were individually injected with hot water extracts of *G. tenuistipitata* and *G. amanasii* at different concentrations. At all the dosages, total hemocyte count, phenoloxidase activity and respiratory burst increased after two days while phagocytic activity and clearance efficacy increased after one day of injection with *G. tenuistipitata.* Also, the survival of shrimps challenged with *V.alginolyticus* was higher in shrimps that received *G. tenuistipitata* or *G. amanasii.* The above investigations revealed that *L. vannamei* that received hot-water extracts of *G. tenuistipitata* or *G. amanasii* had enhanced immunity and increased resistance against *V. alginolyticus* and hence, the hot-water extract of both the algae could be used as immunostimulants for *L. vannamei.*

The total haemocyte count, PO activity, respiratory burst, phagocytic activity and clearance efficacy to *V. alginolyticus* were evaluated after injecting the white shrimp *L. vannamei* with dopamine or noradrenaline at 10^{-8}, 10^{-7}, and 10^{-6} mol/shrimp (Cheng *et al.*, 2005c; Cheng *et al.,* 2006a). The results revealed that shrimps which received dopamine or noradrenaline had increased susceptibility to *V.alginolyticus* infection. Also, the values for different immunological parameters declined in those shrimp that received dopamine or noradrenaline .

In a study to assess the effect of *Sargassum fusiforme* polysaccharide extracts on vibriosis resistance and immune activity of the shrimp, *Fenneropenaeus chinensis,* Huang *et al.* (2006) observed that the oral administration of *Sargassum fusiforme* polysaccharide extracts at an optimal level of 0.5 and 1.0 per cent for 14 d effectively improved vibriosis resistance and enhanced immune activity of shrimps in general.

Mercier *et al.* (2006) subjected juvenile shrimp, *L. vannamei* reared in either outdoor concrete tanks or indoor plastic tanks to a repeated stress induced by daily handling for 4 weeks and compared the immune

response (total haemocyte count, superoxide anion production, and superoxide dismutase activity) with unstressed shrimp. The authors observed no significant differences between stressed and unstressed shrimps raised in either experimental system, suggesting that repeated stress did not affect the immune response.

Sajeevan *et al.* (2006) studied the immunostimulatory effect of marine yeast *Candida sake* S165 in *Fenneropenaeus indicus* by feeding the shrimps for 28 d with varying biomass concentrations of the yeast and observed that 10 per cent *C. sake* in the diet was found to elicit an optimum immune response in shrimps in general.

Wang *et al.* (2006) demonstrated that supplementation of ascorbic acid in enriched live food enhanced the anti-oxidant capacity of shrimp, increasing its defense system that may fight against environmental stress leading to reduced ammonia toxicity.

Cheng *et al.* (2006b) observed that the susceptibility of *M. japonicus* to *V.alginolyticus* correlated with reductions in immune functions like decrease in total haemocyte count, reduction in hyaline cells, PO activity, phagocytic activity and clearance efficacy to *V.alginolyticus* when shrimps were exposed to sulphide at 575 µg/litre or more.

2.4.2 Research on immunomodulators in *Penaeus monodon*

In an experiment to study the *In vitro* effect of microbial cell wall components peptidoglycan (PG), lipopolysaccharide (LPS) and laminarin, Sritunyalucksana *et al.* (1999b) observed increased PO activity in laminarin fed shrimp and decreased antibacterial activity in LPS fed shrimp. The authors also suggested involvement of LPS in mechanisms for both clotting and for antibacterial activity.

Cheng *et al.* (2000) evaluated the immunomodulatory effects of dietary β-1,3-glucan derived from *Schizophyllum commune, in* the brooders of *P. monodon* and observed enhanced phagocytic activity, cell adhesion and superoxide anion production in shrimps.

Immunity enhancement in the shrimp, *P. monodon* by a probiont *Bacillus* spp. was studied by Rengpipat *et al.* (2000). Survival and growth of shrimps fed probiont in 290 d culture trials were better when compared with control shrimps. The phagocytic activity, PO and anti bacterial activity were found to be increased by feeding *Bacillus* S11. Further, survival of shrimps infected with pathogenic *V.haeveyi* was higher in probiont bacteria fed shrimps. The results documented that *Bacillus* S11 provided disease protection by activating both cellular and humoral immune defense functions as well as providing competitive exclusion in the shrimp's gut.

Effect of dietary copper on the non-specific immune responses of juvenile *P.monodon* was investigated by Lee and Shiau (2002). The results revealed that shrimps fed diets supplemented with 10 and 20 mg Cu/Kg had better weight gain, increased feed and protein efficiency, increased total haemocyte count and intracellular superoxide anion production than those fed unsupplemented control diet.

Lee and Shiau (2003) reported that increase in dietary vitamin C levels in the diet improved the respiratory burst response and prevented tissue copper accumulation in *P. monodon* fed with high dietary copper.

Preparation of spent brewer's yeast β-glucans with a potential application as an immunostimulant for *P. monodon* was investigated by Suphantharika *et al.* (2003). *In vitro,* they observed enhanced phenoloxidase activity in the treated shrimp haemolymph when compared to controls without glucan. Also, *in vivo,* an oral

administration of 0.2 per cent glucan in diets for three days revealed increase in the PO activity of the shrimps.

Azad *et al.* (2005) studied the routes of immunostimulation vis-a-vis survival and growth of *P. monodon* post larvae and suggested that the booster dose of immunostimulation, in general, was advantageous in inducing growth and protective response in shrimps. Also, they indicated that in-feed route of administration was more practical as well as productive.

Supamattaya *et al.* (2005) studied the effect of commercially available *Dunaliella* extract on growth performance, health condition, immune response and disease resistance in *P. monodon.* The authors observed higher resistance to WSSV infection and better tolerance to stress induced by low dissolved oxygen condition when *Dunaliella* extract was fed at a dose of 300 mg/ kg feed. However, the shrimps fed 125–300 mg of *Dunaliella* extract/kg diet for 8 weeks showed higher weight gain and survival compared to the control but there was no significant difference in total haemocyte count and phenoloxidase activity among the treatment groups.

Immunostimulatory effect of methanolic extracts of selected Indian immunostimulant herbs (*Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba*) against WSSV infection in *P.monodon* with reference to haematological, biochemical and immunological changes was studied by Citarasu *et al.* (2006). Among the different concentrations of herbal immunostimulant supplemented diets, the shrimps fed on diet containing 800 mg /kg of herbal extract had better survival and reduction in the viral load. Also, better values of haematological, biochemical and immunological parameters were observed in shrimps treated with herbal immunostimulants. The

findings of the study revealed that the application of herbal immunostimulants was effective against shrimp viral pathogens.

Shiau and Jiang (2006) conducted an 8-week feeding trial to determine the dietary zinc requirement and its effect on the non-specific immune responses of juvenile *P. monodon* by providing 7, 17.5, 28, 35, 48, 57, 87 and 127 mg Zinc/kg diet. Shrimp fed diets supplemented with ≥35 mg Zn/kg had greater weight gain than those fed diets with ≤17.5 mg Zn/kg. Both intracellular superoxide anion production ratios and total haemocyte count were better in shrimp fed diets with 35 and 48 mg Zn/kg diet. The immune indicators suggested that an adequate dietary Zn concentration for better nonspecific immune responses in *P. monodon* was about 35–48 mg Zn/kg diet.

Chang *et al.* (2007) investigated the adverse effects of dopamine on the immunity in *P.monodon* by measuring the total haemocyte count, differential haemocyte count, PO activity, respiratory burst, superoxide dismutase activity, phagocytic activity and clearance efficiency to the pathogen *Photobacterium damsela* and concluded that stress-inducing dopamine suppressed the immune system, which in turn increased the susceptibility of *P. monodon* to *P. damsela.*

2.4.3 Environmental factors acting as immunomodulators of shrimp immune system

Cultured shrimp are subjected to climatic changes and changes due to rearing practices that influence the physico-chemical quality of water. Physico-chemical changes of sea water affect the metabolism, growth, moulting and survival that can influence the immune system (Le Moullac and Haffner, 2000). Most research work related to fluctuations in natural environment and immune responses is carried out in the crab,

Carcinus maenas. However, scanty literature is available on the effect of environmental insults on the immune response in shrimp.

It is reported that low oxygen tension hampers the metabolic performances in shrimp and can reduce growth and moulting frequency (Allan and Maguire, 1991) and cause mortality (Madenjian *et al.*, 1987). Crustaceans show several adaptation responses to hypoxia such as reduction of metabolic rate (Hill *et al.*, 1991) and change in osmotic pressure of the haemolymph (Charmantier *et al.*, 1994). Decrease in dissolved oxygen is a common hazard in shrimp culture (Jiang *et al.*, 2005).

The immune response of *P. stylirostris* exposed to severe hypoxia was measured in terms of total haemocyte count, differential haemocyte count, PO activity and respiratory burst (Le Moullac *et al*, 1998). Hypoxia induced a decrease of total haemocyte count which was due to a decrease in semi granular cells and hyaline cells. On the other hand, increased PO activity was related to a reduction of plasma inhibitors regulating the proPO system. There was also a decrease in the total NBT staining, although the activity per cell did not change.

In *P.monodon*, the phagocytic activity of haemocyte was less efficient in oxygen depleted shrimp (Direkbusarakom and Danayadol, 1998). The average clearance efficiency of oxygen-depleted shrimp was approximately 50 per cent less than that in control shrimp. Le Moullac *et al.* (1998) measured *in vitro* the ability of haemocytes after the stress, whereas Direkbusarakom and Danayadol (1998) stimulated first *in vivo* the defenses by injecting the shrimp with a yeast suspension, and in these conditions, plasmatic recognition factors were involved in phagocytosis. The decrease in total haemocyte count in *P. stylirostris* and phagocytosis in *P.monodon* was attributed to low oxygen level in the

pond water which caused an increased susceptibility to infectious diseases.

Jiang *et al.* (2005), in an experiment to study the effect of dissolved oxygen on immune parameters of the white shrimp, *L. vannamei*, observed decreased THC /antibacterial activity and increased PO activity in shrimps exposed to 3.3 and 2.0 mg O_2/litre when compared to control shrimps exposed to 7.5 mg O_2/litre.

Li *et al.* (2006) studied the effects of dissolved oxygen concentration and stocking density on growth and non-specific immunity factors in Chinese shrimp, *Fenneropenaeus chinensis*. The results revealed that dissolved oxygen concentration was one of the key factors affecting shrimps through influencing activities of non-specific immunity, while the stocking density affected the growth performance of shrimp mainly by influencing the activities of enzymes and the interactive effects of dissolved oxygen concentration and stocking density played a crucial role in the production of shrimp.

Water temperature is probably the most important environmental variable because it directly affects metabolism, oxygen consumption, growth, moulting and survival (Chen *et al.*, 1995; Henning and Andreatta, 1998). Temperature has a direct effect on other environmental parameters such as salinity and oxygenation of the water. In the brown shrimp, *P.californiensis*, a temperature increase from 18°C to 32°C affected haemolymph parameters, showing a decrease in total haemolymph proPO at 32°C and an increase of plasmatic protein at 28°C and 32°C (Vargas-Albores *et al.*, 1998).

Cheng *et al.* (2005a) studied the effect of water temperature on the immune response of *L. vannamei* and susceptibility to *V.alginolyticus* and concluded that transfer of shrimp from 27 or 28°C to higher

temperatures (32 and 34°C) reduced their immune capability and resistance to *V.alginolyticus* infection.

The immune response of *P.monodon* and its susceptibility *Photobacterium damselae* under temperature stress was investigated by Wang and Chen (2006). The authors concluded that transfer of *P.monodon* from 26°C to 22°C and 34°C reduced their resistance against *Photobacterium damselae* infection.

In another experiment to understand how stress induced by extreme temperature modulates the immunological behaviour of *Litopenaeus setiferus* males, Pascual *et al.* (2003) used some immune responses as indicators of stress and reported that high temperature caused a reduction in haemocyte proPO activity.

In *P. stylirostris*, the effect of temperature drop from 27°C to 18°C during 24 h on total haemocyte count and PO activity was studied (Le Moullac and Haffner, 2000). It was observed that in shrimp exposed to low temperature, THC dropped by 40 per cent whereas, PO activity increased significantly. However, adaptation phenomena were observed since in the cold season in New Caledonia, when the temperature was around 20°C, the total haemocyte count in *P. stylirostris* was as elevated as in the hot season (Le Moullac and Haffner, 2000).

It is suggested that maximum growth of an organism occurs in an isoosmotic media, since the animal would be expending the minimal amount of energy in osmotic regulation. However, salinity itself has little effect on the metabolic rate of euryhaline shrimp, indicating that the energy required for osmotic regulation may be relatively small. On the other hand, under unhealthy conditions such as viral infections, the stress provoked by high salinity further augments growth retardation produced by the infection (Bray *et al.*, 1990).

The effect of salinity on plasma protein concentration and total haemocytic proPO has been studied by Vargas-Albores *et al.* (1998) in *P. californiensis*. In this study, juvenile shrimps were acclimatized for 20 d at different salinities (28, 32, 36, 40 and 4‰ at 25°C). Total protein levels were not affected, but total proPO increased as salinity increased.

In order to look for technically simple, rapid and low cost stress indicators, Perazzolo *et al.* (2002) evaluated some haemato-imunological parameters in the shrimp *Farfantepenaeus paulensis* submitted to environmental and physiological stress like low salinity, unilateral eyestalk ablation in females and spermatophore extirpation in males. Among the assessed hemato-immunological parameters, the total haemocyte counts and the total serum protein concentration were found to be the the most promising parameters to indicate shrimp stress status.

Effects of mercury on the immune functions have been studied in the fresh water prawn, *Machrobrachium idae* (Victor *et al.*, 1990). The prawns exposed to 1 µg/litre of mercuric chloride over a 30 d period exhibited hyperplastic gill lamellae engorged with haemocytes. It was suggested that the metal could affect haematopoiesis since mercury at a concentration of 50 µg/litre suppressed the circadian rhythmicity of haemocyte numbers.

Effects of short term (96 h) exposure to dissolved heavy metals (mercury, cadmium, lead, copper, chromium and zinc) on the number of circulating haemocytes in the shrimp, *Palaemon elegans* was investigated by Lorenzon *et al.* (2001). Changes in haemocyte counts were determined in relation to time of exposure and metal concentration. It was found that immersion in artificial sea water containing these heavy metals caused a decrease in the haemocyte count during the first 8 h of exposure, although the haemocyte counts returned to initial levels over

the following 16 h of immersion. The greatest decrease in haemocyte numbers was induced by lead, followed by zinc, mercury, chromium, copper and cadmium.

Effect of copper sulfate on the immune response and susceptibility to *V.alginolyticus* in *L.vannamei* was studied by Yeh *et al.*, (2004). Shrimps were challenged with *V.alginolyticus* and then placed in water containing different concentrations of copper. Shrimps exposed to copper for 24 h showed decreased THC, PO activity, phagocytic activity and clearance efficiency as well as increased mortality due to *V. alginolyticus* infection.

Short term (96 h) toxic effects of copper and cadmium at sub-lethal concentrations on the total haemocyte count and serum phenoloxidase activity in *Fenneropenaeus indicus* were investigated in relation to time of exposure and concentration of the metals used (Sharma *et al.*, 2005). It was observed that decrease in haemocyte count and PO activity values in shrimps exposed to metals were rapid and transient. Also, the rapid development of hemocytopenia and decreased PO activity was more conspicuous in case of shrimp exposed to cadmium than those exposed to copper.

Ammonia is known to be very toxic to aquatic animals and can cause impairment in numerous organs (Colt and Armstrong, 1981). In the intensive culture system, ammonia is the commonest toxicant resulting from excretion by cultured animals and ammonification of unconsumed feed (Le Moullac and Haffner, 2000).

An experiment to determine the dose-response effect of ammonia was carried out on shrimp immune response including the study of expression of the proPO and peroxinectin genes in *P. stylirostris* (Le Moullac and Haffner, 2000). The treatment resulted in reduction in the

amount of haemocytes by 15 per cent at 1.5 mg/litre and 50 per cent at 3.0 mg/litre. Concurrently, the amount of transprict encoding proPO and peroxinectin decreased by 60% and 50%, respectively in response to stress.

Immune response of *L. vannamei* and its susceptibility to *V. alginolyticus* under ammonia stress was studied by Liu and Chen (2004). Among the different immune parameters studied, no difference in total haemocyte count was observed among shrimps at different ammonia-N concentrations. PO activity however, decreased when the shrimps were exposed to 5.24 mg/litre ammonia-N and greater after 7 days. It was concluded that ammonia in water caused a depression in the immune response and an increase in mortality of *L. vannamei* from *V. alginolyticus* infection.

In an experiment to study the susceptibility of *L. vannamei* to *V. alginolyticus* under nitrite stress, Tseng and Chen (2004) challenged the shrimp with *V .alginolyticus* and then placed in water containing different concentrations of nitrite. It was observed that nitrite in water caused a depression in the immune ability of *L. vannamei* to *V. alginolyticus* infection together with an increase in super oxide anion production.

In an experiment to examine the effects of harbour dredge spoils on the immune capability of common shrimp, *Crangon crangon*, Smith *et al.* (1995b) observed that the immune capability was adversely affected in shrimps exposed to harbour dredge spoils as indicated by elevation in recoverable haemolymph volume, reduction in total haemocyte count and reduced blood cell phenoloxidase activity.

Propiconzole, a fungicide, injection in shrimp *P. vannamei* induced an increase in respiratory burst on Day 6 following injection where as, on Day 13, a significant dose-dependant decrease of the respiratory burst to

the injected amount of Propiconzole, was observed (Le Moullac and Haffner, 2000).

Immune response of *L.vannamei* and its susceptibility to *Vibrio* infection in relation to moult cycle was studied by Liu *et al.* (2004). It was observed that THC, PO activity, respiratory burst and clearance efficiency were highest in intermoult but lower at post moult stages. Also, the mortality of shrimps injected with *V. alginolyticus* was significantly higher in shrimps at postmoult stage than those at intermoult stage. The authors concluded that *L.vannamei* showed a decrease in resistance to infection due to decrease in immunological values at post moult stage when compared to other stages.

In an attempt to know how starvation level modulates catabolism and its effects on the immune response, Pascual *et al.* (2006) studied juvenile *L. vannamei* that had been starved for varying period after being conditioned on diet containing either maintenance or optimal dietary protein levels and observed a reduction in all the physiological and immunological indicators with starvation. It was suggested that shrimps with good nutritional condition could tolerate starvation until 14 d without modifying the evaluated immune responses.

2.4.4 Bacterial biofilm as immunomodulator in aquaculture

Natural bacterial populations tend to occur as assemblages enmeshed in a polymeric glycocalyx matrix called biofilm to take advantage of the nutrient concentrating effect and to gain protection against predator and toxic agents (Anwar *et al.*, 1984). This protective nature of bacterial biofilms was exploited for the development of an effective oral vaccine for finfish that can resist gastric destruction of epitopes, facilitating improved antigen delivery (Azad *et al.*, 1999). The oral vaccination with biofilm cells of *Aeromonas hydrophila*, a common

fish pathogen, elicited a significantly higher immune response and protection in carps (Azad, *et al.,* 1999). Azad *et al.* (2000) attributed superior antigen delivery to lymphoid tissues for the better performance of the biofilm vaccine as demonstrated by antigen localization using monoclonal antibodies.

In an experiment to enhance growth of common carp, rohu and tilapia through the use of sugarcane bagasse as substrate, Umesh *et al.* (1999) observed higher production of fish when bagasse was supplemented with cattle dung. This higher production of fish was attributed to bacterial biofilm promoted on the substrate which, apart from forming food for zooplankton and fish, contributed to improved water quality by lowering ammonia.

In another experiment, Joice *et al.* (2002) evaluated bacterial biofilm promoted on sugarcane bagasse in nursery for its effect on growth, survival and resistance to *Aeromonas hydrophila,* in hatchlings of common carp, *Cyprinus carpio.* It was observed that common carp grew faster under sugarcane bagasse treatment. The authors reported that fry reared in biofilm enhanced system had higher serum agglutination titre and protection against *Aeromonas hydrophila* compared to those from control and thus indicated the scope for improving the resistance of fish against ubiquitous secondary pathogens through biofilm production.

In a study to analyse the total protein, S-layer protein and LPS of biofilm cells of *A. hydrophila* by SDS-PAGE and to compare with that of planktonic cells, Asha *et al.* (2004) reported absence of S-layer protein and presence of an additional higher molecular weight band of LPS in biofilm cells compared to that in planktonic cells. The authors indicated that changes in the LPS profile might have contributed to the loss of S-layer. They also suggested that the high molecular weight band of LPS

might play a role in the better performance of biofilm oral vaccine by eliciting a protective immune response.

Published works on the effect of bacterial biofilm on growth and immune response in shrimp culture system are scarce. Thompson *et al.* (2002) conducted studies to test the usefulness of biofilms in reducing the levels of ammonia and phosphate of rearing system water, and as food source for the shrimp *Farfantepenaeus paulensis*. The biofilm mass consisted of diatoms (*Amphore, Campylopyxis, Navicula, Sinedra, Hantschia and Cylindrotheca*) and filamentous cyanobacteria (*Oscillatoria* and *Spirulina*). The authors reported that pinnate diatoms and filamentous cyanobacteria were responsible for the largest uptake of ammonia from the water. It was suggested that the presence of biofilm lead to reduced exportation of phosphorous and to a higher output of nitrate + nitrite, instead of ammonia.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 *In vitro* development of biofilm and free cells of *Vibrio alginolyticus* for oral immunostimulation in shrimp

3.1.1 Bacterial isolate and maintenance of pure culture

A virulent isolate of *Vibrio alginolyticus* isolated from infected *Penaeus monodon* was identified using Gram's stain and routine biochemical tests. The isolate was used for the development of free and biofilm cells. The isolate was grown in 1.5 per cent Trypticase Soya Broth (TSB) supplemented with 2 per cent NaCl and harvested by centrifugation at 1500 x g for 10 min. The cell pellet was suspended in sterile TSB (1.5 per cent W/V + 2 per cent NaCl) with 15 per cent glycerol. Later it was aliquoted into 1.5 ml microcentrifuge tubes and stored at -20°C for future use in various experiments.

For use, the culture was revived on nutrient agar slants (supplemented with 2 per cent NaCl) over night at room temperature and stored at 4°C until further use.

3.1.2 Standardization of nutrient requirement for optimum biofilm production

In vitro model of biofilm development of *Vibrio alginolyticus* was according to Umesh *et al.* (1999) for the development of biofilm cells of *Aeromonos hdrophila.* Six concentrations of TSB (0.025, 0.05, 0.1, 0.15, 0.2 and 0.25 per cent) were prepared in triplicate in 250 ml flasks and supplemented with 2 per cent NaCl. Chitin flakes (0.3 per cent, W/V) were added to each flask and autoclaved at 115°C for 30 min at 15 lb pressure. Sterile TSB (1.5 per cent W/V) supplemented with 2 per cent NaCl was inoculated with *Vibrio alginolyticus* isolate maintained in nutrient agar slants and incubated over night at room temperature. The

flasks containing chitin suspension were inoculated with 1 ml of the bacterial suspension after adjusting the OD (640) at 0.9. The flasks were incubated at room temperature for 24 h with agitation at 120 strokes/min on a mechanical shaker for 6 h. After incubation, the supernatant was carefully decanted into sterile 250 ml flasks without disturbing the flakes. The flakes were washed 3 times with sterile Phosphate Buffered Saline (PBS) (pH 7.2) by gently swirling the flasks three times in order to remove unbound cells. Chitin flakes with biofilm cells were collected in 10 ml PBS in a 50 ml sterile centrifuge tube and agitated for 4 min in a vortex shaker to dislodge the cells. The supernatant (0.1 ml) containing the biofilm cells was serially diluted 10 fold in sterile physiological saline. From each dilution, 0.1 ml was transferred to three Trypticase Soya Agar (TSA) (supplemented with 2 per cent NaCl) plates and was spread uniformly. The plates were incubated for 24 h at room temperature. Number of colonies were counted and expressed as cfu/g chitin flakes. The TSB concentration which gave highest cfu/g chitin flakes of biofilm cells was considered for further studies.

The supernatant containing planktonic cells was centrifuged at 1500 X g for 15 min and washed thrice with sterile PBS. The pellet was resuspended in 10 ml PBS and serially diluted. From each dilution, 0.1 ml was placed on TSA plates and spread uniformly. After incubation for 24 h at room temperature, the colonies were counted and expressed as cfu/ml.

3.1.3 Growth kinetics of biofilm and free cells of *Vibrio alginolyticus*

Six flasks in triplicate containing 0.3 per cent (W/V) suspension of chitin flakes in 0.15 per cent TSB (as per the concentration determined in 3.1.2) supplemented with 2 per cent NaCl were inoculated with one ml

of the inoculum (OD 0.9) of *V.alginolyticus* in log phase and incubated for five days at room temperature with 6 h of agitation per day at 120 strokes/min on a mechanical shaker. At the end of 1, 2, 3, 4, and 5 days, the flasks were removed. Biofilm and free cells were harvested as in 3.1.2 and the mean cfu was determined.

3.1.4 Heat inactivation of biofilm and free cells

Three-day-old biofilm cells were harvested as above and placed in a water bath at 60, 70 and 80°C for 10 and 20 minutes. Twenty four hour culture of free cells grown in 1.5 per cent TSB was harvested and placed in water bath at 60°C for 10 min. The samples plated on TSA were incubated for 24 h and per cent inactivation was determined.

3.1.5 Formalin inactivation of biofilm and free cells

Three-day-old biofilm cells were harvested as above and finally incubated in sterile PBS (pH 7.4) containing 1, 5 and 10 per cent formalin for 12, 24 and 48 h at room temperature. Twenty four hour culture of free cells grown in 1.5 per cent TSB was harvested and suspended in PBS containing 0.5 per cent formalin and incubated for 24 h at room temperature. Samples from both biofilm and free cells plated on TSA were incubated for 24 h at room temperature. Per cent inactivation of cells was determined.

3.1.6 Protein profile of biofilm and free cells of *Vibrio alginolyticus*

To study the antigenic expression in biofilm and free cells, protein profile was analyzed using SDS-PAGE according to Laemmli (1970). Three-day-old biofilm of *V.alginolyticus* on chitin flakes was harvested by vortex mixing in PBS with 1 mM phenylmethylsulphonylfloride (PMSF). The biofilm mass was pelleted after centrifugation at 84730 X g for 30 min at 4°C. One day old free cells of *V.alginolyticus* were centrifuged at

1500 X g for 10 min. The cells were harvested and washed thrice in sterile PBS (pH 7.2). The samples were solubilized in reducing buffer and vortexed well. The solutions were then boiled at 100°C for 1 min and spun at 5000 rpm for 2 min to settle down the debris. The supernatants (20 µl) were loaded on to 4.5 per cent stacking gel and resolved in a 15 per cent separating gel at 150 V in a mini gel electrophoretic system. The run was stopped when the dye reached the bottom of the gel. After electrophoresis, the resolved protein bands were visualized in coomassie brilliant blue (0.1 per cent (W/V) in 40 per cent methanol and 10 per cent glacial acetic acid solutions). They were stained for 1 h followed by destaining in 40 per cent methanol and 10 per cent glacial acetic acid till the background was clear. The molecular weights were determined after comparison with protein molecular weight standards (Sigma, USA).

3.2 Preparation of experimental diet

Required quantity of heat inactivated biofilm cells on chitin flakes were mixed uniformly with standard commercial shrimp feed with the help of a binder (Protogel, Orgavet Pharma, Vijayawada, India). Required concentration of free cells was mixed in the feed with help of the binder. Autoclaved chitin flakes were mixed in the control feed using the binder.

3.3 Standardization of dose of biofilm cells for use as immunostimulant in *Penaeus monodon* juveniles

3.3.1 Source and maintenance of shrimp

Juvenile shrimps (1.7±0.3 g) were procured from the Mathsyafed Prawn Hatchery, Kannur, Kerala and transported to the laboratory. The shrimps were acclimatized in 1 ton circular FRP tanks containing 500 litres of chlorinated sea water (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90 per cent saturation) for 15 d (Fig.1). The shrimps were fed with standard commercial shrimp feed twice daily at 10 per cent

of body weight during this period. The uneaten feed and waste were siphoned out twice daily and 20 per cent water was replaced.

After the acclimatization period, the shrimps were divided into different groups and subjected to various treatments as shown in Table 1. The experiment was conducted in 3 stages. Shrimps were maintained in circular plastic tubs (Fig.2) containing 70 litres of chlorinated seawater (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90 per cent saturation) and fed with biofilm incorporated feed at 8 AM daily at 5 per cent of body weight for two weeks. It was confirmed that the shrimps consumed all the feed within 3 h. At 3 PM, standard feed without biofilm cells were fed at 5 per cent of body weight. The control shrimps were fed twice daily at 10 per cent of body weight with standard feed containing autoclaved chitin flakes and binder. The waste was siphoned out before feeding and 3 h after feeding. Observations were recorded on Day zero, 7 and 14.

Table 1. Experimental design for standardization of dose of biofilm cells

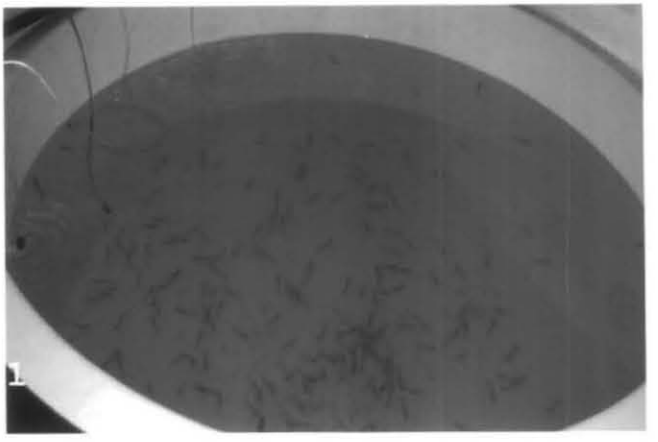
Experiments	Treatment groups		Replicates
Experiment-1	Control (Normal feed + chitin flakes + binder)	Treatment 1 (10^8 cfu/g shrimp/day)	Triplicate with 30 shrimp in each replicate
Experiment-2	Control (Normal feed + chitin flakes + binder)	Treatment 2 (10^9 cfu/g shrimp/day)	Triplicate with 30 shrimp in each replicate
Experiment-3	Control (Normal feed + chitin flakes + binder)	Treatment 3 (10^{10} cfu/g shrimp/day)	Triplicate with 30 shrimp in each replicate

Fig.1. Photograph to show acclimatization of *Penaeus monodon* juveniles in the laboratory in circular FRP tanks

Fig.2. Photograph to show rearing of shrimps for experimentation in circular plastic tubs

Fig.3. Photograph to show collection of haemolymph from the heart of shrimp

Fig.4. Photograph to show determination of moult stage: Note the closely adhered epidermis to the cuticular buds (arrow) during intermoult stage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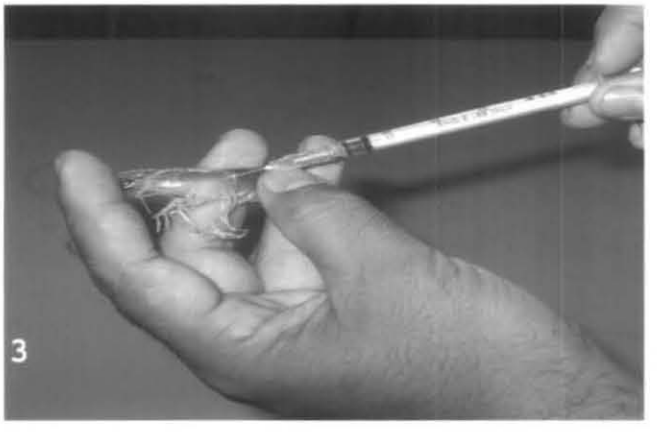
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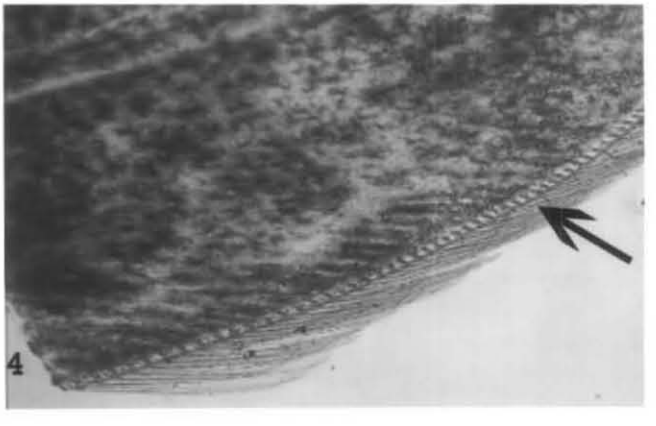
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3.3.2 Determination of dose of biofilm cells for immune activation in *P. monodon*

3.3.2.1 Preliminary studies

A preliminary study to understand whether biofilm cells have immune stimulating effect in *P. monodon* was conducted with two groups of 10 shrimp (1.6±0.3 g) each in duplicate. The shrimps were maintained in plastic tubs containing 70 litres of sea water (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90 per cent saturation). The first group was fed with biofilm cells of *V.alginolyticus* on chitin flakes through feed at the rate of 10^8 cfu/g shrimp/d for 7 d. The second group which was given standard shrimp feed containing autoclaved chitin flakes served as control. On 7th day, haemolymph samples were collected from individual shrimp and subjected to total haemocyte count and phenoloxidase activity as described under subsequent sub-headings.

3.3.2.2 Immune response studies

In order to study the immune functions in *P. monodon* fed with various concentrations of biofilm cells, haemolymph was collected directly from the heart using a tuberculin syringe fitted to a 26 gauze needle (Fig.3) on 0, 7th and 14th day from representative samples of shrimps from each group in their inter moult stage. The moult stage was determined by cutting the tip of the uropod and observing it under the microscope. Absence of epidermal retraction and the epidermis closely adherent to cuticular buds was considered as intermout stage (Fig.4). Wherever the haemolymph collected was insufficient, haemolymph from 2 or more shrimps were pooled. For enumeration of haemocyte count, antibacterial activity and phenoloxidase activity, 300 µl of haemolymph was collected in 700 µl of marine anticoagulant solution.

3.3.2.2.1 Enumeration of total haemocyte count (THC)

A drop of the haemolymph-anticoagulant mixture was dispensed on a Neubaur slide and the cells were allowed to settle for few seconds. The cells were counted in all five large squares. The haemocyte count was expressed as cells/ml of haemolymph (Liu and Chen, 2004).

3.3.2.2.2 Phenoloxidase activity assay

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) according to Liu and Chen (2004). Diluted haemolymph was centrifuged at 700 x g at 4°C for 10 min. After discarding the supernatant, the pellet was rinsed, resuspended gently in cacodylate-citrate buffer, and then centrifuged again. The pellet was then resuspended in 600 µl cacodylate buffer and 100 µl of the cell suspension was incubated with 50 µl of trypsin (1 mg/ml), which served as an elicitor, for 10 min at 20°C. Fifty µl of L-DOPA (3 mg/ml) was added and 800 µl of cacodylate buffer was added 5 min later. The optical density at 490 nm was measured using Varion Carry-50 spectrophotometer and recorded. The PO activity was expressed as δ (490)/50 µl of haemolymph. The control solution which consisted of 100 µl of cell suspension, 50 µl cacodylate buffer (to replace trypsin) and 50 µl of L-DOPA was used for background phenoloxidase activity. The background phenoloxidase activity which ranged from 0·02 to 0·08, was subtracted from the phenoloxidase activity of shrimps for all test conditions.

3.3.2.2.3 Antibacterial activity assay

Antibacterial activity was assayed according to Smith *et al.* (1995a) and Sung *et al.* (1996) with modifications. *V. alginolyticus* was grown to log phase in 1.5 per cent TSB supplemented with 2 per cent NaCl. The

cells were harvested by centrifuging at 400 x g for 10 min followed by washing twice with sterile PBS (pH 7.4). Finally, the cells were resuspended in PBS at a concentration of 1 x 10^7 cfu/ml. Ten µl of the prepared suspension of the test bacterium was incubated with 80 µl of haemocyte suspension prepared in section 3.3.2.2.2 and 10 µl of sterile 1.5 per cent TSB (supplemented with 2 per cent NaCl) in duplicate wells of a microtitre plate. The final concentration of bacterial cells was 2 x 10^6/ml. For controls, 90 µl of 1.5 per cent TSB supplemented with 2 per cent NaCl was incubated with 10 µl of bacterial suspension in duplicate wells. A third pair of duplicate wells containing 80 µl of cell suspension, 10 µl of PBS and 10 µl of 1.5 per cent TSB supplemented with 2 per cent NaCl served as contamination control. The contents of the plate were mixed well. Fifty µl from each well was transferred on to preformed TSA plates in duplicate and spread. The microtitre plates and the TSA plates were incubated at 36°C for 24 h. After 24 h, colonies on the TSA plates were counted. Fifty µl from each well of the microtitre plate was transferred on to preformed TSA plates in duplicate and spread. After incubating the plates for 24 h at 36 °C, the colonies were counted. The plate counts thus obtained were expressed as the Survival Index (SI) as per the following formula:

$$SI = \frac{\text{cfu at time } t_t}{\text{cfu at time } t_0}$$

where t_0 is the initial cfu and t_t is the final cfu. SI values above one indicated bacterial growth, whereas, SI values less than one indicated an antibacterial effect.

3.3.2.2.4 Estimation of total haemolymph protein

For estimation of total haemolymph protein, method of Lowry *et al*, (1956) was adopted. Haemolymph (50 µl) collected in 80 per cent ethanol was centrifuged at 700 X g for 5 min. The pellet was dissolved in 1N

NaOH and subjected to protein estimation. The values were expressed as mg protein/ml of haemolymph.

3.4 Comparative studies on the immune functions of *Penaeus monodon* exposed to biofilm and free cells

Thirty shrimps (1.6±0.3 g) were maintained in plastic tubs with 70 litres sea water (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90 per cent saturation) in three groups with triplicate tubs in each group. Group one was given 10^9 cfu biofilm cells on chitin flakes/g shrimp through the feed for 14 d. Group two was given 10^9 cfu free cells/ g shrimp through the feed for 14 d. Shrimps in group three which received autoclaved chitin flakes alone through the feed served as control. Haemolymph was collected on Day zero and 14 and subjected to total haemocyte count, phenoloxidase activity, total haemolymph protein and antibacterial activity as explained in 3.3.2.2. The immunological values in different groups were enumerated.

3.5 Resistance of *P.monodon* fed with biofilm and free cells to common pathogens

3.5.1 Resistance of *P.monodon* to *Vibrio alginolyticus*

3.5.1.1 Isolation and maintenance of pure culture of pathogenic *Vibrio alginolyticus*

The bacterium was isolated from the haemolymph of infected *P.monodon* on 1.5 per cent TSB supplemented with 2 per cent NaCl and confirmed by biochemical tests. The isolate was preserved in aliquots in 1.5 per cent TSB supplemented with 2 per cent NaCl and 10 per cent glycerol.

3.5.1.2 Determination of LD_{50}

LD_{50} of *P.monodon* was determined following the method of Selvin and Lipton (2003). Ten juvenile *P.monodon* (1.7±0.4 g) were maintained in 8 plastic tubs containing 70 litres sea water, in duplicate. Prior to the experiments, shrimps were selected randomly and checked for the presence of bacterium. *Vibrio alginolyticus* isolated from infected *P.monodon* and maintained on slant was activated on 1.5 per cent TSB supplemented with 2 per cent NaCl and sub cultured in fresh media for 18 h on a shaker. The cells were centrifuged at 400 x g for 15 min. The cell pellets were washed thrice with sterile PBS, resuspended in PBS and serially diluted in normal saline for enumeration by plating on TSA (supplemented with 2 per cent NaCl) plates. Shrimps were injected with cells at 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 cfu in 0.1 ml normal saline at the ventral side on the second segment using 1 ml tuberculin syringe fitted with 26 gauze hypodermic needle. Control shrimps in duplicate received 0.1 ml of normal saline only. Mortality of shrimp was recorded at the end of 6, 12, 24, 48, 72, 96, 120, 144 and 168 h post injection. Haemolymph of moribund shrimps was used for re-isolation of bacterium using TCBS agar (Himedia, Mumbai). The LD_{50} for 24 h was calculated.

3.5.1.3 Challenge studies

Juveniles of *P.monodon* (1.7 ±0.4) were maintained in plastic tubs with 70 litres seawater (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90per cent saturation) in four groups. Each group was maintained in triplicate with 30 shrimp in each replicate. First group was fed for 14 d with feed incorporated with biofilm cells of *V.alginolyticus* at a dose rate of 10^9 cfu/g/d for 14 d. The second group was fed with feed incorporated with free cells of *V.alginolyticus* at the same dose as biofilm cells. Third group was given normal feed containing chitin flakes and binder. Fourth group was given normal

shrimp feed. The virulent isolate of *V. alginolyticus* was reactivated in 1.5 per cent TSB. Twenty four hour culture of the bacteria in log phase was harvested by washing thrice in sterile PBS (pH 7.2). On 15th day, each shrimp in groups 1, 2 and 3 was injected with 1 x 10^6 cfu of bacterial sample in 0.1 ml of normal saline at the second abdominal segment with the help of a 26 gauze needle fitted to a tuberculin syringe. Each shrimp in fourth group received 0.1 ml of normal saline. Three shrimps from a replicate in each group were removed at 3, 6, 12, 24, 48, 72, 96 and 120 h after challenge for enumeration of *V.alginolyticus* in the haemolymph. Haemolymph (0.1 ml) was aseptically collected at the specified time period from individual shrimp into 0.9 ml of sterile anticoagulant solution. The haemolymph-anticoagulant mixture was centrifuged at 700 x g for 10 min and 0.1 ml of the supernatant was transferred and spread on to triplicate TSA plates. The plates were incubated at room temperature for 24 h and cfu/ ml of haemolymph was calculated. Mortality in remaining two replicates was observed for five days and Relative Per cent Survival (RPS) was calculated as per the following formula:

$$RPS = 1 - \frac{\text{Per cent mortality in biofilm/ free cell group}}{\text{Per cent mortality in control group}} \times 100$$

The dead shrimps and shrimps from which haemolymph was collected were preserved in 10 per cent neutral buffered formalin for histopathology. For histopathology, the tissues were processed, embedded in paraffin wax and the sections (3 μ) were stained with haematoxylene and eosin and histologic Gram's stain of Brown and Brenn method (Luna, 1969). In the case of H & E stained sections, number of haemocytic nodules (maximum 20) in a cross section of abdomen was considered to classify the severity of lesions; 13-20 haemocytic nodules of various size- grade A; 7-12 nodules in a section - grade B; and 1-6 nodules in a section- grade C.

3.5.2 Resistance of *P. monodon* to White Spot Syndrome Virus

3.5.2.1 Purification and preparation of virus sample for challenge studies

The virus from infected *P.monodon* was purified according to Xie *et al.* (2005). Infected tissues except hepatopancreas were collected in a beaker on ice bath. Ten grams of infected tissues were homogenized in 500 ml of TNE buffer and then centrifuged at 3500 x g for 5 min at 4°C. After filtering through nylon net (400 mesh), the supernatant was centrifuged at 30,000 x g for 30 min at 4°C. The upper loose pellet was rinsed carefully and the lower white pellet was suspended in 10 ml TM buffer. After centrifugation at 30,000 x g for 20 min at 4°C the pellet was resuspended and kept in 1 ml TM buffer containing 0.1 per cent NaN_3.

3.5.2.2 Challenge studies

Juveniles of *P.monodon* (1.7 ±0.4) were maintained in plastic tubs with 70 litres seawater (salinity 30±1‰; pH 7.6±0.2; temperature 26±0.2°C; DO >90per cent saturation) in four groups. Each group was maintained in triplicates with 30 shrimp in each replicate. The first group was fed for 14 d with feed incorporated with biofilm cells of *V.alginolyticus* at a dose rate of 10^9 cfu/g/day for 14 d. The second group was fed with feed incorporated with free cells of *V.alginolyticus* at the same dose as biofilm cells. The third group was given normal feed containing chitin flakes and binder. Fourth group was given normal shrimp feed. On 15th d, each shrimp in group 1, 2 and 3 was injected 50 µl of virus sample at the 2nd abdominal segment. The shrimps in group 4 were injected with 50 µl of TM buffer. Three shrimps from one tub in each group were collected every 24 h and tested for WSSV using Immunodot kit (Department of Aquaculture, College of Fisheries, KVAFSU, Mangalore, India) as per the protocol supplied along with the

kit. Shrimps from remaining two tubs from each group were observed for 5 d and mortality was recorded. RPS was calculated as explained in *3.5.1.3.* The dead shrimps were immediately preserved in 10 per cent neutral buffered formalin and subjected to histopathology. The tissues were processed, embedded in paraffin wax and sections of 3 μ thickness were stained with haematoxylene and eosin (Luna, 1969) and immunoperoxidase for confirming WSSV infection.

Immunoperoxidase staining was carried out according to Anil, et al. (2002). The sections were dewaxed in xylene and hydrated to water. After washing with PBS (containing 0.3 per cent tween), sections were treated with 1 per cent Bovine Serum Albumin (BSA) for 30 min and again washed with PBS. Then the sections were allowed to react with monoclonal antibody against WSSV for 45 min followed by washing in PBS. The sections were then reacted with Horse Radish Peroxidase conjugated goat antimouse IgG (1 in 200 in 1 per cent BSA in PBS) for 30 min before washing with PBS. Then the sections were treated with Diaminobenzidine (Sigma, Bangalore)(0.6 mg/ml in 50 mM tris buffer containing 150 mM NaCl and 0.03 per cent hydrogen peroxide) for 5 min. After washing with tap water, the sections were counter stained with 1 per cent eosin for 3 min. They were dehydrated, cleared with xylene and mounted using DPX. Dark brown coloration in tissue was considered as positive reaction.

Severity of lesions under H & E staining was graded as grade A, B and C according to Sudha (1997). If more than 50 per cent cells in the cuticular epithelium contained intranuclear inclusion bodies, the lesion was considered as grade A; 50-5 per cent cells containing inclusion bodies considered grade–B and less than 5 per cent cells containing inclusion bodies as grade-C.

3.6 Statistical analysis

All data were expressed as Mean±SE. Mean values of nutrient requirement for optimum biofilm formation, growth kinetics and preliminary immune response studies were statistically analysed by One-way-ANOVA with tukey post-tests using GraphpadPrism (Version:4) for Windows, GraphPad Software, San Diego, California, USA. The mean values obtained during standardization of biofilm doses, comparative immune response studies of biofilm and free cell groups and bacterial enumeration of haemolymph of various experimental groups were statistically analyzed by performing Two-Way-ANOVA with Bonferroni Post-tests using GraphpadPrism (Version:4) for Windows, GraphPad Software, San Diego, California, USA. The LD_{50} (24 h) was calculated by employing non linear fit of transformation with Boltzmann Sigmoidal Equation using GraphpadPrism (Version: 4).

RESULTS

4. RESULTS

4.1 *In vitro* development of biofilm and free cells of *Vibrio alginolyticus*

4.1.1 Characteristics of the bacterial isolate

The bacterial isolate was identified as *Vibrio alginolyticus* based on morphological and biochemical characteristics. Features of the isolate based on biochemical tests are given in Table 2.

4.1.2 Development of biofilm and free cells of *Vibrio alginolyticus* for oral immunostimulation in shrimp

4.1.2.1 Nutrient requirement for optimum biofilm production

Among six nutrient concentrations studied, significantly highest cfu of biofilm cells was obtained in 0.15 per cent TSB supplemented with 2 per cent NaCl. Growth of biofilm and free cell bacteria in different nutrient concentrations is shown in Table 3 and Fig.5.

4.1.2.2 Growth kinetics of biofilm and free cells of *Vibrio alginolyticus*

Mean cfu of biofilm and free cells at different incubation time are shown in Table 4. Highest cfu/g chitin flakes was obtained after 3 d of incubation which was significantly ($P<0.001$) higher than the cfu values with incubation periods of 1, 2, 4, and 5 days. Highest cfu/ml of free cells was obtained after 48 h of incubation. The growth kinetics of biofilm and free cells of the bacterial isolate are shown in Fig. 6.

Table 2. Characteristics of *Vibrio alginolyticus* isolated from infected *Penaeus monodon* used *for in vitro* biofilm production

Gram staining	+
Shape	Short rod
Growth on TCBS	Yellow
Luminescence	-
Swarming	+
Oxidase production	+
Catalase production	+
Acid/ gas production	
Glucose	Acid
Sucrose	Acid
Mannitol	Acid
Maltose	Acid
Sorbitol	-
Lactose	-
Galactose	-
Arabinose	Acid
Decarboxylase of:	
Arginine	-
Lysine	+
Ornithine	+
Growth in peptone with NaCl (per cent):	
0	-
1	+
3	+
6	+
8	+
10	+

Table 3. Nutrient requirement for optimum production of biofilm cells of *Vibrio alginolyticus* (mean ±SE, n=3)

TSB concentration (per cent)	Biofilm (cfu/g chitin flakes)	Free cells (cfu/ml)
0.025	$6.81 \times 10^8 \pm 8.03 \times 10^7$	$1.82 \times 10^8 \pm 1.29 \times 10^7$
0.05	$8.60 \times 10^8 \pm 9.29 \times 10^7$	$2.10 \times 10^8 \pm 1.21 \times 10^7$
0.1	$5.22 \times 10^9 \pm 1.12 \times 10^9$	$3.13 \times 10^9 \pm 1.32 \times 10^9$
0.15	$8.57 \times 10^9 \pm 2.03 \times 10^8$	$3.56 \times 10^9 \pm 1.35 \times 10^9$
0.2	$5.96 \times 10^8 \pm 1.55 \times 10^8$	$2.23 \times 10^9 \pm 5.36 \times 10^8$
0.25	$5.00 \times 10^8 \pm 1.88 \times 10^8$	$3.10 \times 10^{10} \pm 5.69 \times 10^9$

Table 4. Kinetics of biofilm and free cells of *Vibrio alginolyticus* incubated for five days (mean ±SE, n=3)

Days of incubation	Biofilm cells (cfu/g chitin flakes)	Free cells (cfu/ml)
1	$5.87 \times 10^9 \pm 1.33 \times 10^8$	$6.10 \times 10^9 \pm 4.51 \times 10^8$
2	$6.60 \times 10^{10} \pm 1.20 \times 10^{10}$	$2.70 \times 10^{12} \pm 7.55 \times 10^{11}$
3	$4.47 \times 10^{11} \pm 2.40 \times 10^{10}$	$3.80 \times 10^{11} \pm 2.31 \times 10^{11}$
4	$4.60 \times 10^{10} \pm 1.26 \times 10^{10}$	$3.30 \times 10^9 \pm 8.74 \times 10^8$
5	$3.26 \times 10^{10} \pm 2.77 \times 10^9$	$1.79 \times 10^8 \pm 8.20 \times 10^7$

Table 5. Heat inactivation of 3 day old biofilm and free cells of *V.alginolyticus*

Biofilm cells			Free cells		
Temp (°C)	**Time (min)**	**Per cent inactivation**	**Temp (°C)**	**Time (min)**	**Per cent inactivation**
60	10	91.26	60	10	100
60	20	96.76			
70	10	99.66			
70	20	99.99			
80	10	100			

Table 6. Formalin inactivation of 3 day old biofilm cells of *V.alginolyticus*

Concentration of formalin (Per cent)	**Per cent inactivation at**		
	12 h	**24 h**	**48 h**
1	-	-	92.22
5	96.22	99.66	99.99
10	99.92	100	

Fig.5. Growth of biofilm and free cells of *Vibrio alginolyticus* under different nutrient concentrations

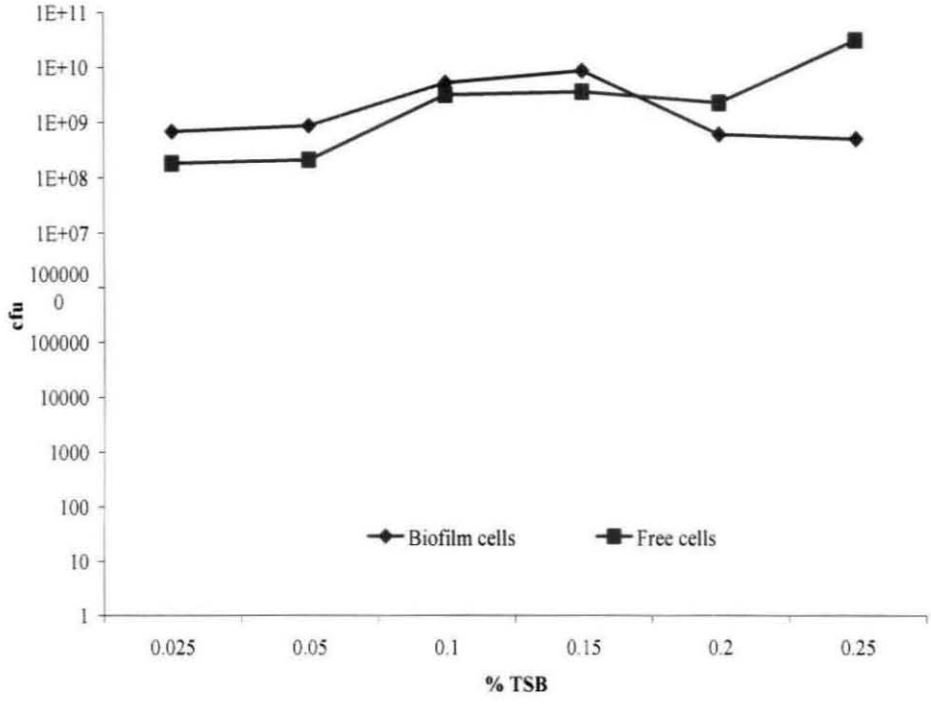
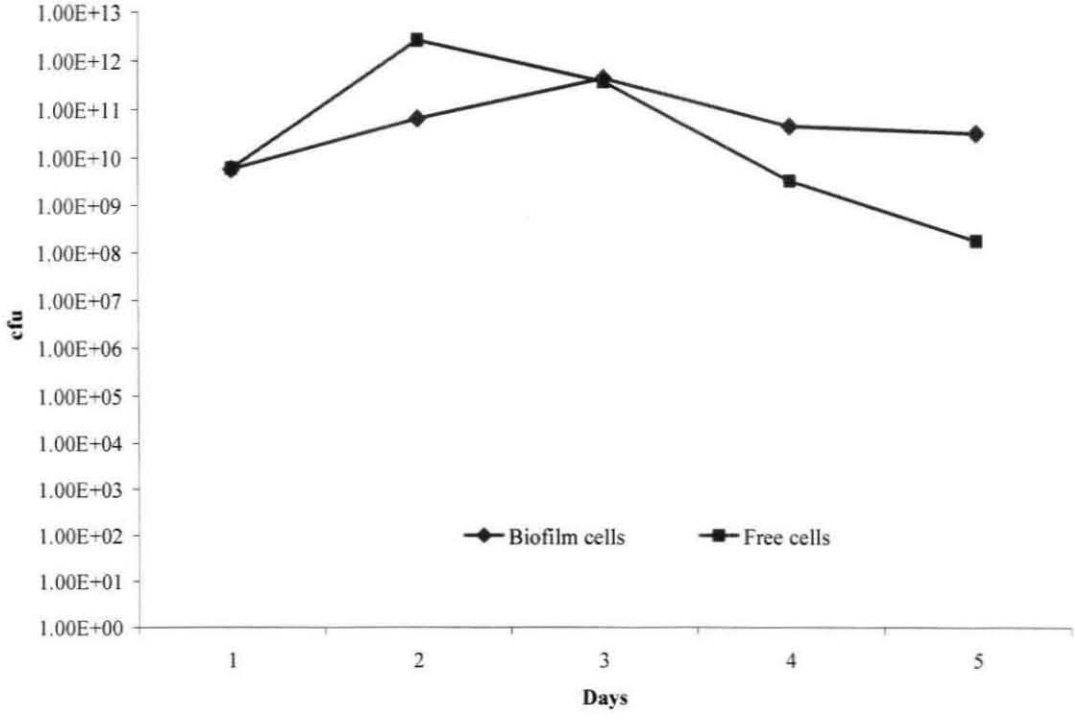


Fig.6. Growth kinetics of *Vibrio alginolyticus* in biofilm and planktonic phase



4.1.2.3 Inactivation of *V.alginolyticus* biofilm and free cells

Inactivation of *V.alginolyticus* biofilm and free cells was carried out at varying temperature regimen and formalin concentrations. Complete inactivation of 3- day- old biofilm cells was at 80°C for 10 min whereas it was observed at 60°C for 10 min for planktonic cells (Table 5). In formalin treated biofilm cells, complete inactivation was achieved with 10 per cent formalin for 24 h (Table 6) while for planktonic cells it was with 0.5 per cent formalin for 24 h.

4.1.2.4 Protein profile of biofilm and free cells of *V.alginolyticus*

SDS – PAGE of 3-day-old biofilm and free cells of *V.alginolyticus* revealed difference between them. A total of 22 proteins were found with free cells. Biofilm cells had an additional expression of 3 proteins of 45, 50 and 55 kDa and repression of 4 proteins of 31, 44, 47, and 53 kDa when compared to free cells (Fig.7).

4.2 Standardization of dose of biofilm cells for use as immunostimulant in *Penaeus monodon*

4.2.1 Preliminary studies

Mean total haemocyte count and phenoloxidase activity of biofilm fed and control shrimps are shown in Table 7. Mean values of both the immune parameters were significantly ($P<0.001$) higher in biofilm fed group on Day 7 when compared to control shrimps (Figs. 8 & 9).

Fig.7. SDS-PAGE of biofilm and free cells of *V.alginolyticus*: green bars indicate repressed proteins; red bars indicate expressed proteins

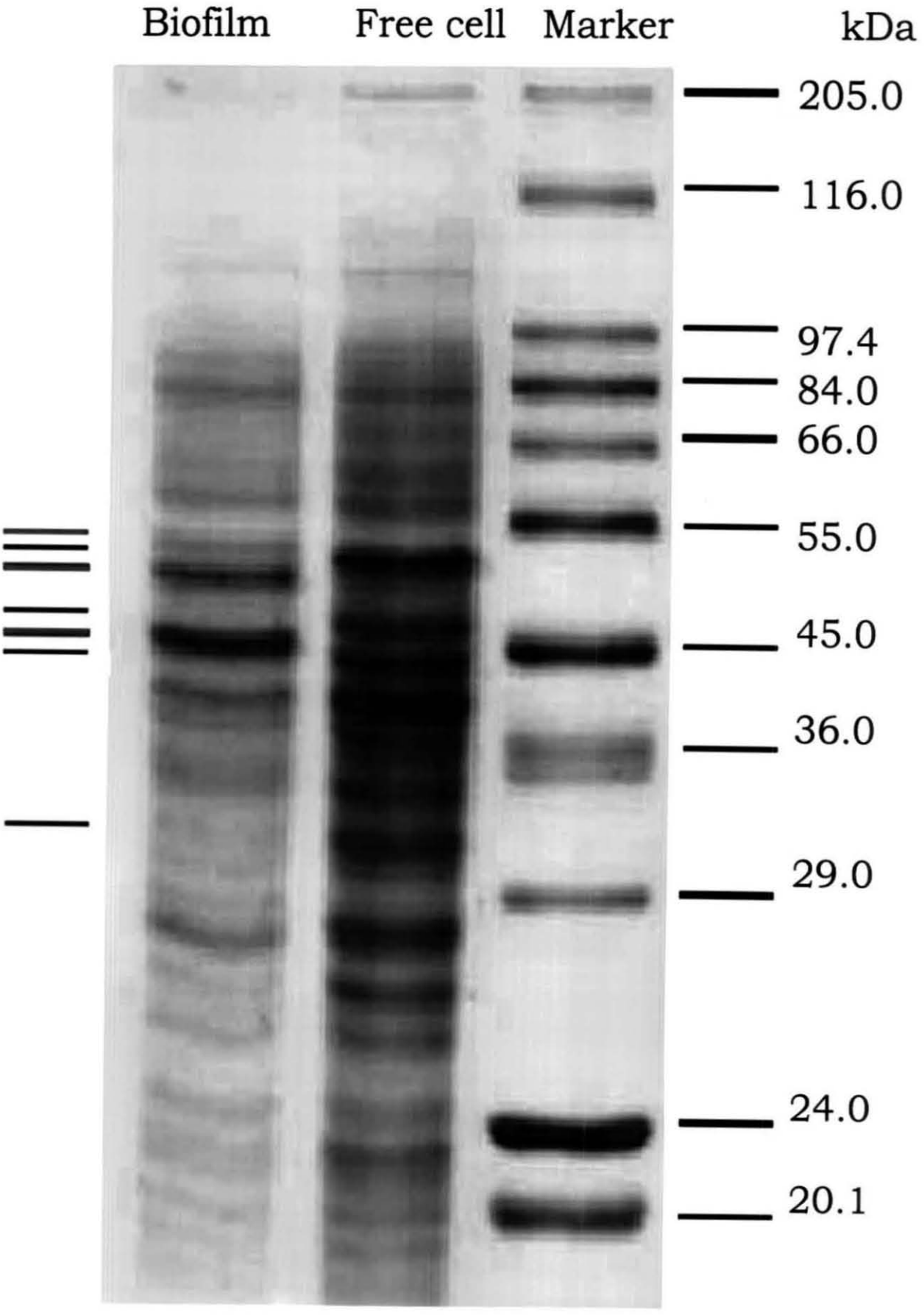

Biofilm
Free cell
Marker
kDa
205.0
116.0
97.4
84.0
66.0
55.0
45.0
36.0
29.0
24.0
20.1

Table 7. Preliminary studies showing mean±SE of total haemocyte count (THC) and phenoloxidase (PO) activity of shrimp exposed to 10^8 cfu/g biofilm cells

Immune parameters	Days	Mean ±SEM	
		Control	Biofilm
THC	0	68.2±2.62[a]	66.7±2.79[a]
(x 10^5 cells/ml)	7	63.2±2.49[a]	91.1±3.03[b]
n= 7			
PO	0	0.046±0.000[a]	0.043±0.001[a]
(Δ OD/450)	7	0.047±0.001[a]	0.065±0.005[b]
n=10			

Superscripts with different alphabets have significant difference ($P<0.001$)

Fig.8. Preliminary studies showing total haemocyte count of shrimp after feeding them with 10^8 cfu/g/day biofilm cells for 7 d (vertical bars indicate standard error)

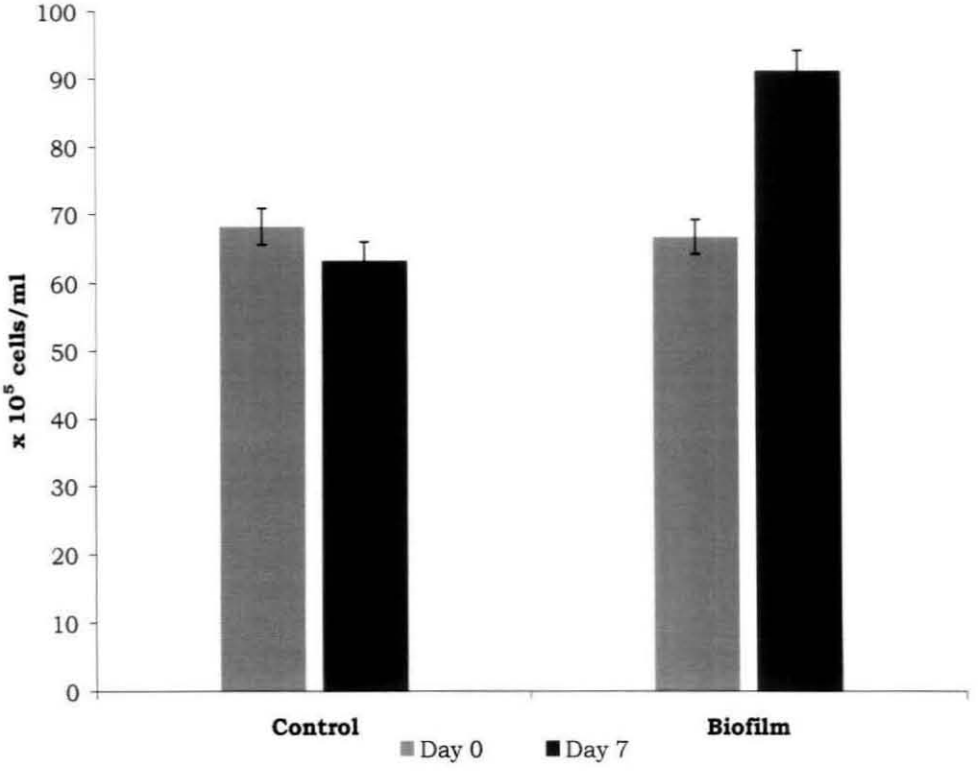
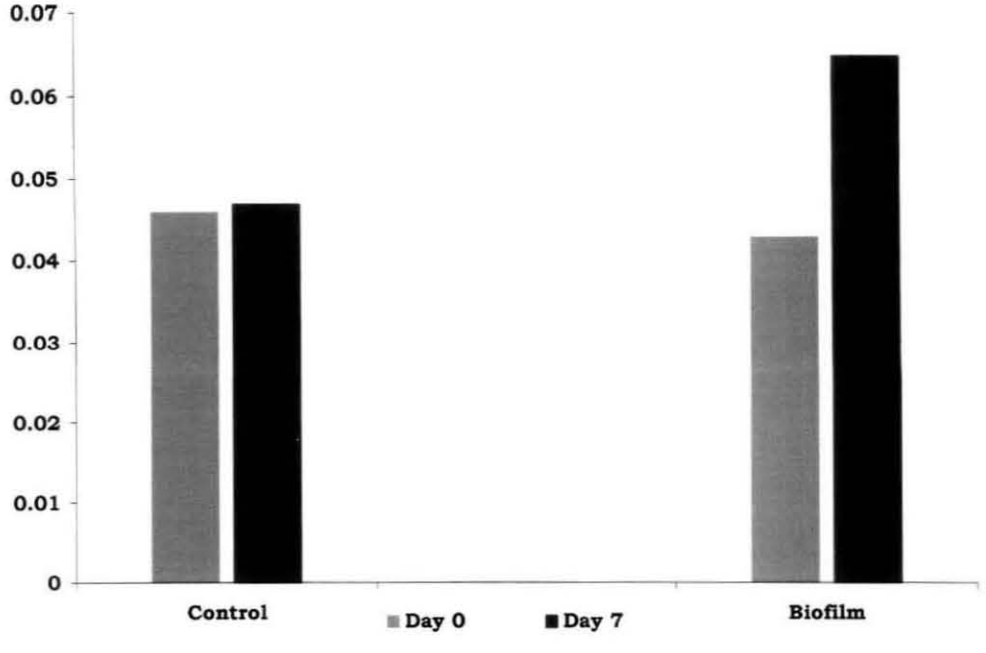


Fig.9. Preliminary studies showing PO activity of shrimp after feeding them with with 10^8 cfu/g/day biofilm cells for 7 d (vertical bars indicate standard error)



4.2.2 Immune response of shrimps fed with different concentrations of biofilm cells

Mean values of total haemocyte count, phenoloxidase activity, total haemolymph protein and anti-bacterial activity of shrimps fed with different concentrations of biofilm cells as compared between doses is shown in Table 8. Mean values of various immune parameters of shrimps exposed to different concentrations of biofilm cells as compared between days of treatment is shown in Table 9.

4.2.2.1 Total haemocyte count

The total haemocyte count of control shrimps and shrimps fed with different biofilm cell concentrations are depicted in Fig. 10. The haemocyte counts of shrimps exposed to 10^8 and 10^9 cfu biofilm cells were significantly ($P<0.05$) higher than control shrimps on Day 7. The values in shrimp exposed to 10^{10} cfu were also significantly ($P<0.01$) higher than the values observed in control shrimps. On Day 14, the cell counts of shrimps fed with 10^8 ($P<0.05$), 10^9 ($P<0.001$) and 10^{10} cfu ($P<0.001$) were significantly higher than that of control shrimps. The THC values of shrimps which were given 10^9 and 10^{10} cfu biofilm cells were significantly ($P<0.001$) higher than the values in shrimp fed with 10^8 cfu biofilm cells. In shrimps treated with 10^8 cfu biofilm cells, the haemocyte counts on Day 7 and 14 were significantly ($P<0.05$) higher than the counts observed on Day zero. In shrimps exposed to 10^9 and 10^{10} cfu biofilm cells also the counts on Day 7 ($P<0.01$) and Day 14 ($P<0.001$) were significantly higher than the counts observed on Day zero. The THC values of shrimps exposed to 10^9 and 10^{10} cfu were significantly ($P<0.001$) higher on Day 14 when compared to Day 7. No significant difference in the values was observed between 10^9 and 10^{10} cfu groups.

4.2.2.2 Phenoloxidase (PO) activity

Mean PO activity of shrimps exposed to different treatment groups are depicted in Fig.11. The mean PO values of the shrimps exposed to 10^8, 10^9 and 10^{10} cfu biofilm cells were significantly ($P<0.01$) higher than the control shrimps on Day 7. The PO values on Day 14 were significantly higher in shrimps exposed to 10^8 ($P<0.01$), 10^9 ($P<0.001$) and 10^{10} ($P<0.001$) cfu biofilm cells when compared to control shrimps. Significantly higher values in shrimps exposed to 10^9 ($P<0.05$) and 10^{10} ($P<0.01$) cfu biofilm cells were observed on Day 7 when compared to shrimps exposed to 10^8 cfu. Also, significantly ($P<0.001$) higher PO activity was seen in groups exposed to 10^9 and 10^{10} cfu on Day 14 than the PO activity observed in 10^8 cfu exposed group on the same day. No significant ($P<0.05$) difference in PO activity was observed between the groups exposed to 10^9 and 10^{10} cfu biofilm cells. Significantly higher PO activity was observed on Day 7 compared to Day zero in shrimps exposed to 10^8 ($P<0.01$), 10^9 ($P<0.01$) and 10^{10} ($P<0.001$) cfu biofilm cells. On Day 14, PO activity values were significantly ($P<0.001$) higher than the values on Day zero in all the experimental groups. PO values observed on Day 14 were significantly higher than the values observed on Day 7 in shrimps exposed to 10^8 ($P<0.01$), 10^9 ($P<0.001$) and 10^{10} ($P<0.001$) cfu biofilm cells.

4.2.2.3 Total haemolymph protein

Mean protein values of shrimps exposed to different treatment groups are depicted in Fig.12. The mean protein values of shrimps exposed to 10^9 and 10^{10} cfu biofilm cells were significantly ($P<0.001$) higher than the control shrimps on Day 14. Significantly higher protein values were observed on Day 14 in shrimps exposed to 10^9 ($P<0.001$) and 10^{10} ($P<0.05$) cfu biofilm cells than those exposed to 10^8 cfu. No significant ($P<0.05$) difference was noticed in protein values between the

Table 8. Mean±SE values of total haemocyte count (THC), phenoloxidase activity (PO), total protein (TP) and anti- bacterial activity (AB) of shrimps exposed to different concentrations of biofilm cells (dose 1:10^8 cfu, dose 2:10^9 cfu, dose 3:10^{10} cfu): comparison between groups

Immune parameters	Days	Mean ± SE			
		Control	Dose-1	Dose-2	Dose-3
THC (x 10^5 cells/ml) N= 12	0	74.1±1.06	72.6±1.00	72.2±1.13	73.4±9.11
	7	72.8±1.17	87.7±3.68 a*	88.9±7.51 a*	92.9±7.78 a**
	14	71.6±1.04	87.7±1.66 a*	138.0±5.54 a*** b***	137.0±6.33 a*** b***
PO (Δ OD/450) N=9	0	0.058±0.001	0.059±0.002	0.054±0.001	0.055±0.001
	7	0.058±0.001	0.067±0.003 a**	0.076±0.002 a** b*	0.089±0.002 a** b**
	14	0.055±0.002	0.090±0.002 a**	0.133±0.003 a*** b***	0.132±0.003 a*** b***
TP (mg/ml) N=9	0	41.8±3.5	37.1±2.4	43.9±2.8	45.7±2.4
	7	47.7±2.5	46.5±2.6	52.5±2.8	55.0±2.6
	14	46.5±3.4	50.7±2.1	66.8±1.1 a*** b***	61.8±2.9 a*** b*
AB (SI) N=10	0	1.02±0.01	0.97±0.01	0.99±0.01	0.98±0.01
	7	0.98±0.01	0.97±0.01	0.77±0.02 a*** b***	0.72±0.02 a*** b***
	14	0.95±0.01	0.85±0.02 a*	0.54±0.02 a*** b***	0.50±0.02 a*** b***

a = dose-1, dose-2 and dose-3 compared with control
b = dose-2 and dose-3 compared with dose-1
* =P<0.05; ** = P<0.01; *** = P<0.001

Table 9. Mean±SE values of total haemocyte count (THC), phenoloxidase activity (PO), total protein (TP) and anti- bacterial activity (AB) of shrimps exposed to different concentrations of biofilm cells (dose 1:10^8 cfu, dose 2:10^9 cfu, dose 3:10^{10} cfu): comparison between days

Immune parameters	Days	Mean ± SE			
		Control	Dose-1	Dose-2	Dose-3
THC (x 10^5 cells/ml) n= 12	0	74.1±1.06	72.6±1.00	72.2±1.13	73.4±9.11
	7	72.8±1.17	87.7±3.68 a*	88.9±7.51 a**	92.9±7.78 a**
	14	71.6±1.04	87.7±1.66 a*	138.0±5.54 a*** b***	137.0±6.33 a*** b***
PO (Δ OD/450) n=9	0	0.058±0.001	0.059±0.002	0.054±0.001	0.055±0.001
	7	0.058±0.001	0.067±0.003	0.076±0.002 a**	0.089±0.002 a***
	14	0.055±0.002	0.090±0.002 a*** b**	0.133±0.003 a*** b***	0.132±0.003 a*** b***
TP (mg/ml) n=9	0	41.8±3.5	37.1±2.4	43.9±2.8	45.7±2.4
	7	47.7±2.5	46.5±2.6	52.5±2.8 a*	55.0±2.6 a*
	14	46.5±3.4	50.7±2.1 a**	66.8±1.1 a** b**	61.8±2.9 a** b*
AB (SI) n=10	0	1.02±0.01	0.97±0.01	0.99±0.01	0.98±0.01
	7	0.98±0.01	0.97±0.01	0.77±0.02	0.72±0.02
	14	0.95±0.01	0.85±0.02 a** b**	0.54±0.02 a*** b***	0.50±0.02 a*** b***

a = Day 7 and Day 14 compared with day 0
b = Day 14 compared with Day 7
* =P<0.05; **= P<0.01; *** = P<0.001

groups exposed to 10^9 and 10^{10} cfu biofilm cells. On Day 7, the values observed in shrimps exposed to 10^9 cfu were significantly ($P<0.05$) higher than the values on Day zero. On Day 14, significantly ($P<0.01$) higher protein values were observed in all the treatment groups when compared to zero day values. Higher protein values were observed on Day 14 than on Day 7 in shrimps exposed to 10^9 ($P<0.01$) and 10^{10} ($P<0.05$) cfu biofilm cells.

4.2.2.4 Antibacterial activity

Mean antibacterial activity values of shrimps exposed to different treatment groups are depicted in Fig. 13. The mean survival index values of shrimps exposed to 10^9 and 10^{10} cfu biofilm cells were significantly ($P<0.001$) higher than the values observed in control shrimps on Day 7 and 14. In shrimps exposed to 10^8 cfu, the values were significantly ($P<0.05$) higher on Day 14 when compared to control shrimps. Significantly ($P<0.001$) higher survival index values were observed in shrimps exposed to 10^9 and 10^{10} cfu than those exposed to 10^8 cfu on Day 7 and 14. No significant ($P<0.05$) difference was noticed in survival index values between the groups exposed to 10^9 and 10^{10} cfu biofilm cells. The values on Day 14 were significantly higher than the values observed on Day zero and 7 in shrimps exposed to 10^8 ($P<0.01$), 10^9 ($P<0.001$) and 10^{10} ($P<0.001$) cfu biofilm cells.

4.2.3 Comparative studies on the immune functions of shrimp exposed to biofilm and free cells of *Vibrio alginolyticus*

Mean values of total haemocyte count, phenoloxidase activity, total haemolymph protein and antibacterial activity of shrimps exposed to biofilm and free cells are shown in Table 10.

Fig.10. Mean THC of shrimp exposed to different biofilm doses (vertical bars indicate standard error; Dose-1:10^8 cfu; Dose-2:10^9 cfu; Dose-3:10^{10} cfu)

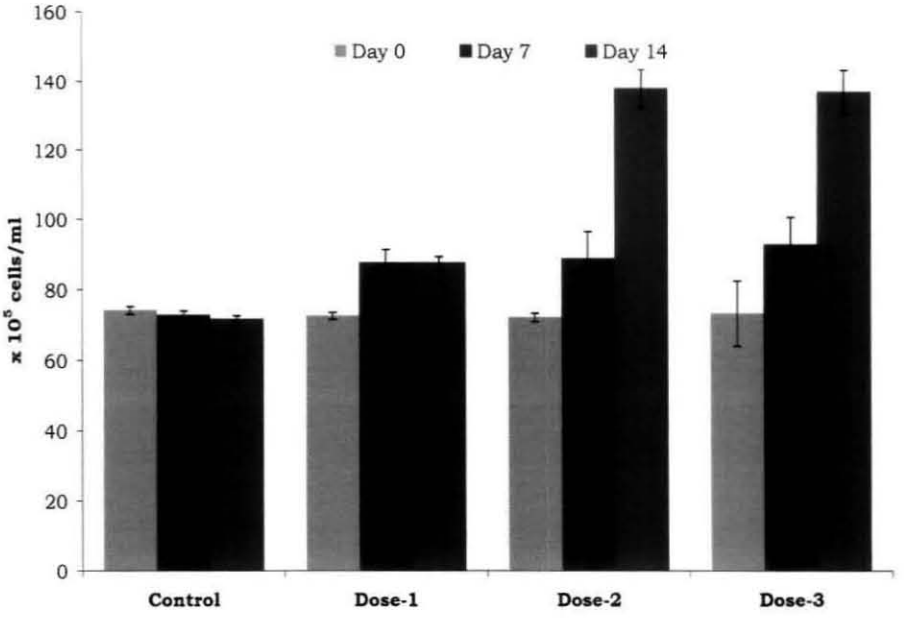


Fig.11. Mean PO activity of shrimp exposed to different biofilm doses (vertical bars indicate standard error; Dose-1:10^8 cfu; Dose-2:10^9 cfu; Dose-3:10^{10} cfu)

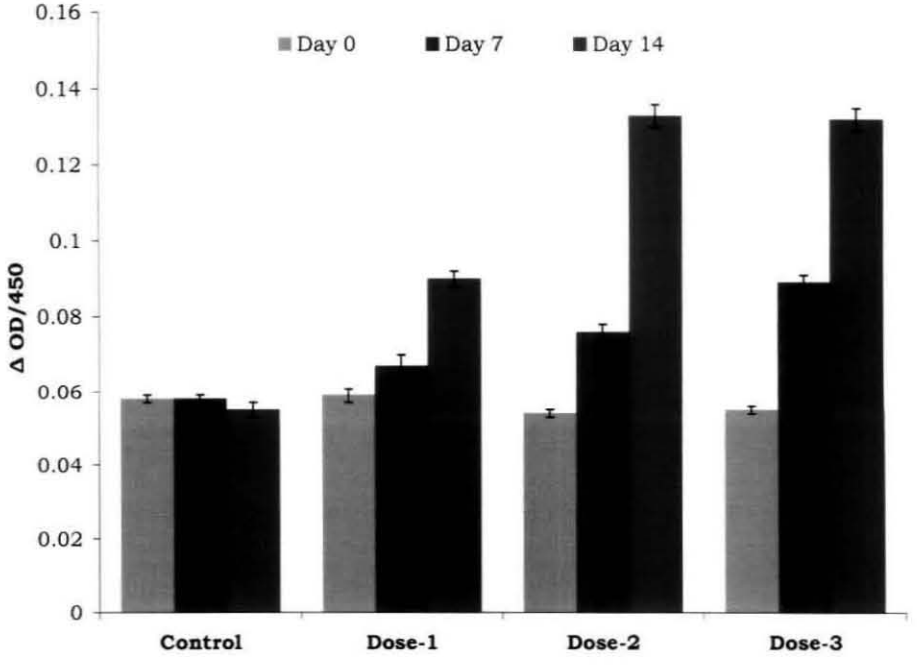


Fig.12. Mean protein values of shrimp exposed to different biofilm doses (vertical bars indicate standard error; Dose-1:10^8 cfu; Dose-2:10^9 cfu; Dose-3:10^{10} cfu)

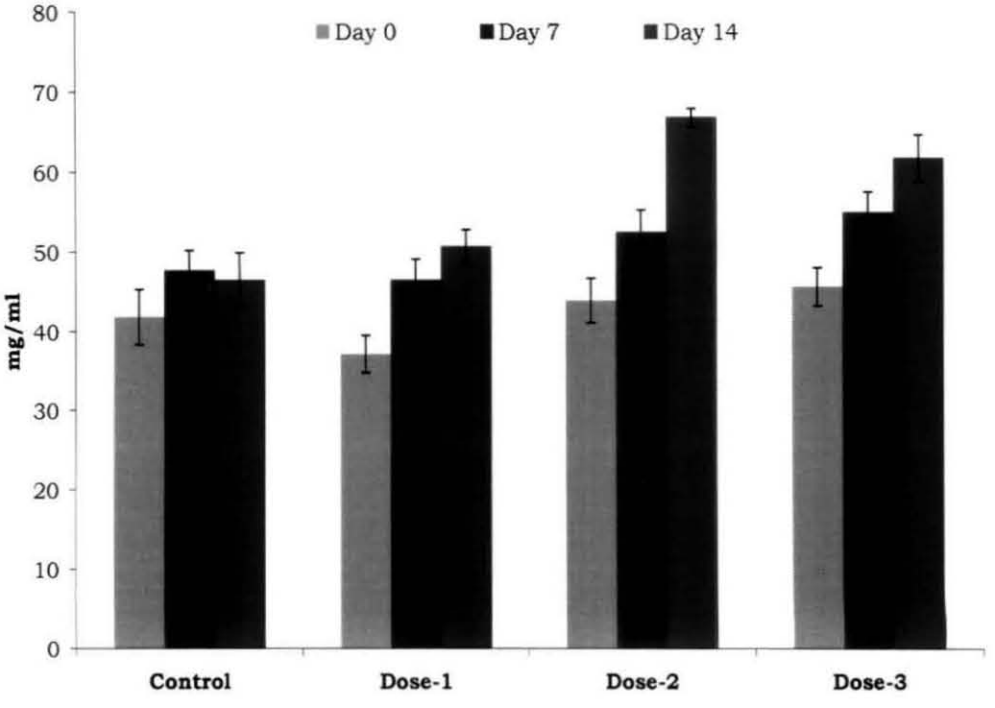
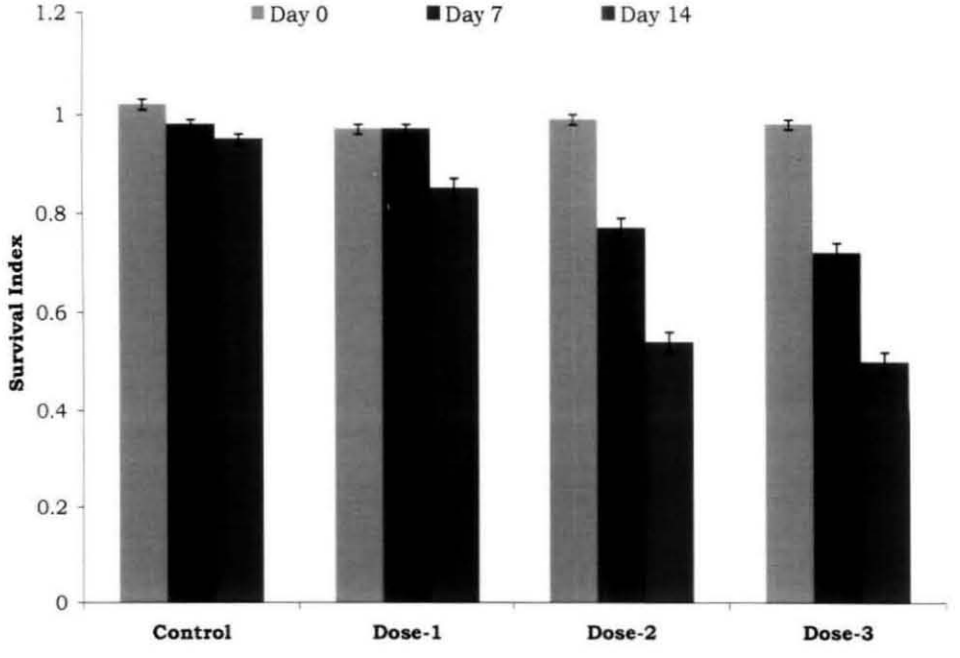


Fig.13. Mean SI of shrimp exposed to different biofilm doses (vertical bars indicate standard error; Dose-1:10^8 cfu; Dose-2:10^9 cfu; Dose-3:10^{10} cfu)



4.2.3.1 Total haemocyte count

Mean haemocyte counts of shrimps subjected to different treatments are depicted in Fig.14. Haemocyte counts of both free and biofilm treated shrimps were significantly ($P<0.001$) higher than control shrimps on Day 14. The cell counts were significantly ($P<0.001$) higher in biofilm treated shrimps than those fed with free cells on Day 14.

4.2.3.2 Phenoloxidase activity

Mean PO values of shrimps subjected to different treatments are depicted in Fig.15. The PO values of both free and biofilm treated shrimps were significantly ($P<0.001$) higher than control shrimps on Day 14. The values were significantly ($P<0.001$) higher in biofilm treated shrimps than free cell treated shrimps on Day 14.

4.2.3.3 Total haemolymph protein

Mean total protein of shrimps subjected to different treatments are depicted in Fig.16. The protein values were significantly ($P<0.001$) higher in biofilm treated shrimps than free cell treated or control shrimps on Day 14.

4.2.3.4 Antibacterial activity

Mean survival index values of shrimps subjected to different treatments are depicted in Fig.17. Values of both free cell and biofilm treated shrimps were significantly ($P<0.001$) higher than control shrimps on Day 14. The values were significantly ($P<0.001$) higher in biofilm treated shrimps than free cell treated shrimps on Day 14.

Table 10. Comparison of the mean±SE values of total haemocyte count (THC), phenoloxidase activity (PO), total protein (TP) and antibacterial activity (AB) of shrimps exposed to 10^9 cfu/g shrimp of biofilm and free cells of inactivated *Vibrio alginolyticus* (n=9; P<0.001)

a = Biofilm and free cell groups compared with control

Immune parameters	Days	Mean ±SE		
		Control	Free cell	Biofilm cell
THC (x 10^5 cells/ml)	0	73.3±1.42	72.1±2.05	74.8±1.00
	14	71.8±2.08	105.0±7.4 a	165.0±15.90 a b
PO (Δ OD/450)	0	0.066±0.001	0.069±.003	0.066±0.002
	14	0.062±0.002	0.089±0.004 a	0.177±0.006 a b
AB (SI)	0	1.02±0.01	0.99±0.03	1.03±0.01
	14	0.99±0.03	0.78±0.02 a	0.55±0.04 a b
TP (mg/ml)	0	42.8±2.6	43.6±2.1	50.6±3.6
	14	40.7±2.4	47.8±3.4	70.7±4.0 a b

b = Bofilm group compared with free cell group

Fig.14. Comparison of mean total haemocyte count of shrimps exposed to 10^9 cfu of biofilm and free cells of *Vibrio alginolyticus* (vertical bars indicate standard error)

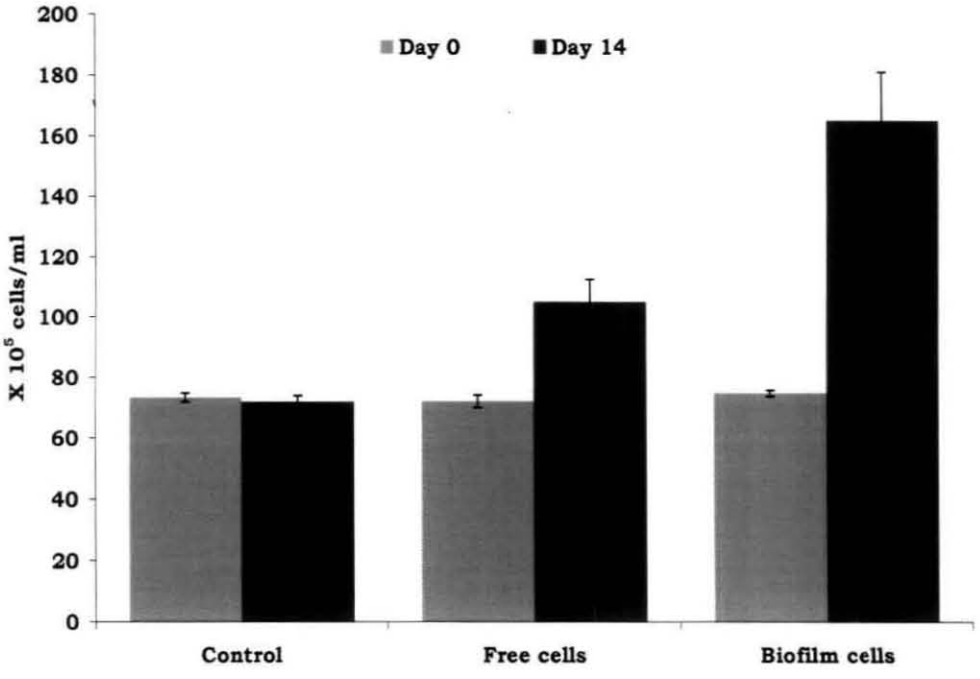


Fig.15. Comparison of mean PO activity of shrimps exposed to 10^9 cfu of biofilm and free cells of *Vibrio alginolyticus* (vertical bars indicate standard error)

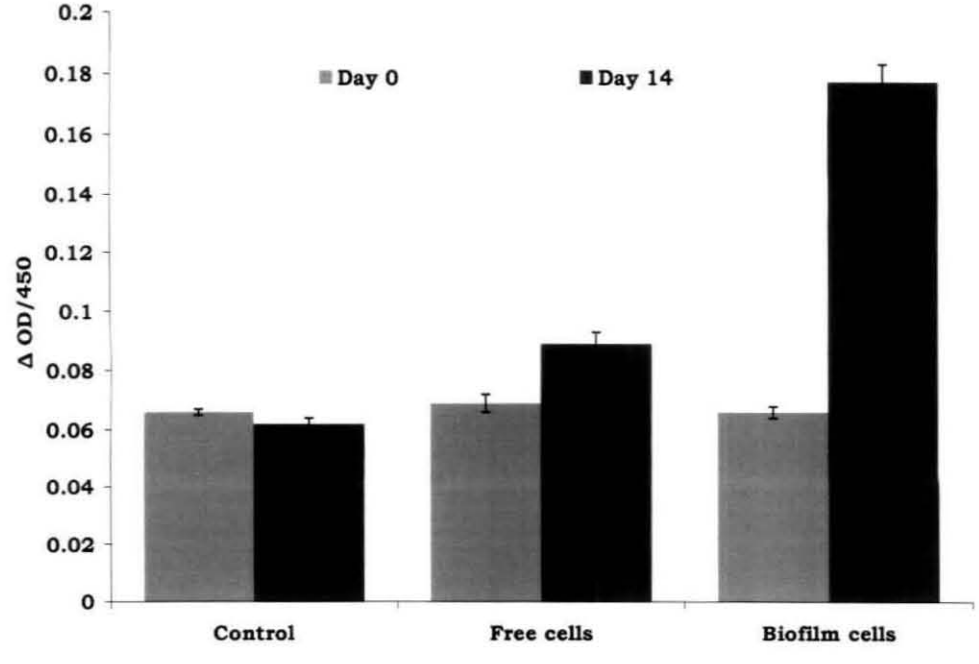


Fig.16. Comparison of mean protein values of shrimps exposed to 10^9 cfu of biofilm and free cells of *Vibrio alginolyticus* (vertical bars indicate standard error)

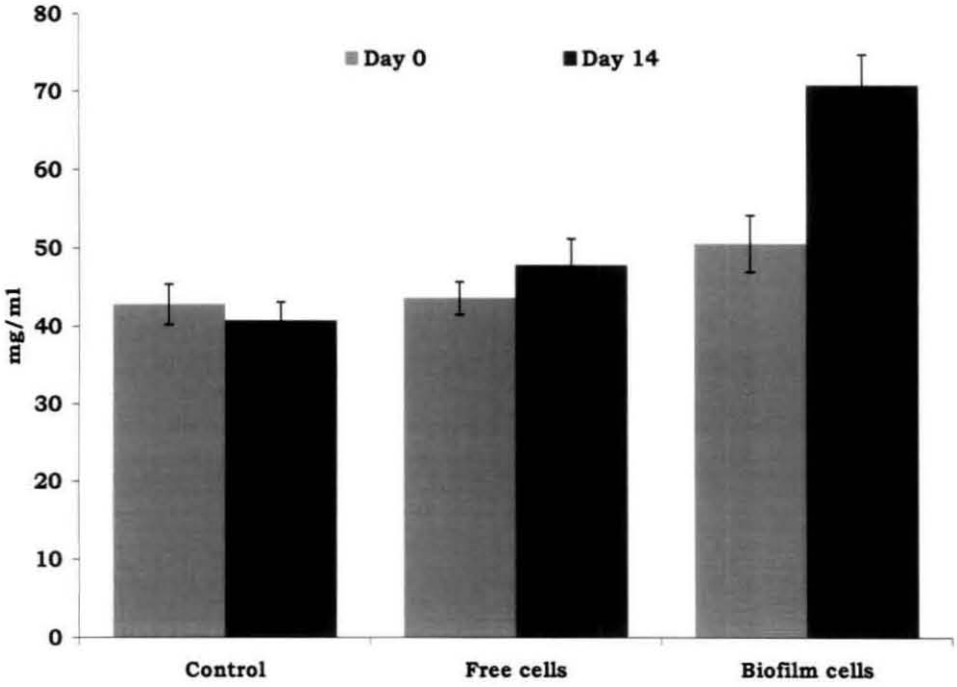
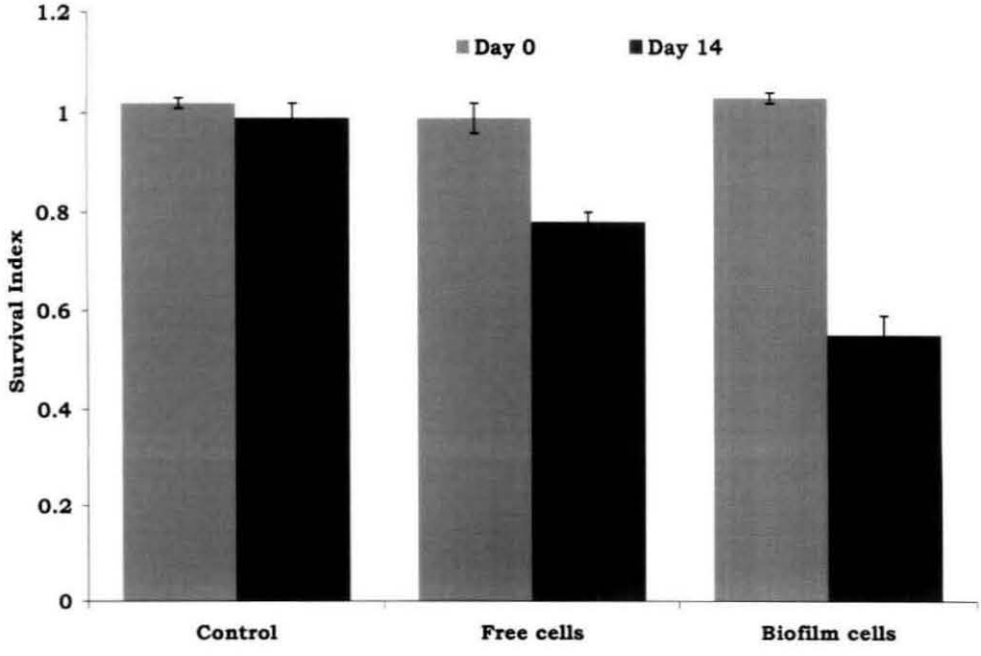


Fig.17. Comparison of mean SI values of shrimps exposed to 10^9 cfu of biofilm and free cells of *Vibrio alginolyticus* (vertical bars indicate standard error)



4.3 Resistance of *P.monodon* juveniles exposed to biofilm and free cells of *Vibrio alginolyticus* to common pathogens

4.3.1 Resistance of *P.monodon* juveniles to pathogenic *Vibrio alginolyticus*

4.3.1.1 Determination of LD_{50}

At higher bacterial cell concentrations (10^9 and 10^8cfu/shrimp), all the shrimps died before 24 h (Table 11). No mortality was observed within 7 days post infection at 10^3 cfu. The LD_{50} value extrapolated from the graph was 3.12×10^7 cfu/shrimp (Fig.18).

4.3.1.2 Enumeration of *V.alginolyticus* in haemolymph

Mean cfu of bacteria in haemolymph of shrimps in control, free cell and biofilm treated groups are shown in Table 12. The bacterial counts (cfu/ml haemolymph) in free cell group was significantly lower than control shrimps at 24 h ($P<0.01$) and 72 to 120 h ($P<0.001$). The cfu in biofilm group was significantly ($P<0.001$) lower than control shrimps from 24 h onwards. The cfu in biofilm group was significantly lower than that observed in free cell group at 48 h ($P<0.001$), 72 h ($P<0.05$), 96 h ($P<0.001$) and 120 h ($P<0.05$). No bacteria could be isolated from haemolymph of shrimps which were administered sterile saline.

4.3.1.3 Relative per cent survival

Relative Per cent Survival (RPS) of shrimps challenged with pathogenic *V.alginolyticus* is depicted in Table 13. The relative per cent was highest (62.1) in biofilm fed group compared to free cell treated shrimp (20.1) at the end of the experiment (120 h).

Table 11. Pathogenicity of *Vibrio alginolyticus* to *Penaeus monodon*

Dose (cfu/shrimp)	Per cent cumulative mortality at (h)								
	6	**12**	**24**	**48**	**72**	**96**	**120**	**144**	**168**
10^9	40	100	100	100	100	100	100	100	100
10^8	40	90	60	100	100	100	100	100	100
10^7	20	40	40	100	100	100	100	100	100
10^6	0	20	30	100	100	100	100	100	100
10^5	0	20	0	60	80	100	100	100	100
10^4	0	0	0	0	0	20	20	20	20
10^3	0	0	0	0	0	0	0	0	0

Fig.18. LD_{50} (24 h) studies: Log cfu of *V.alginolyticus* Vs per cent mortality

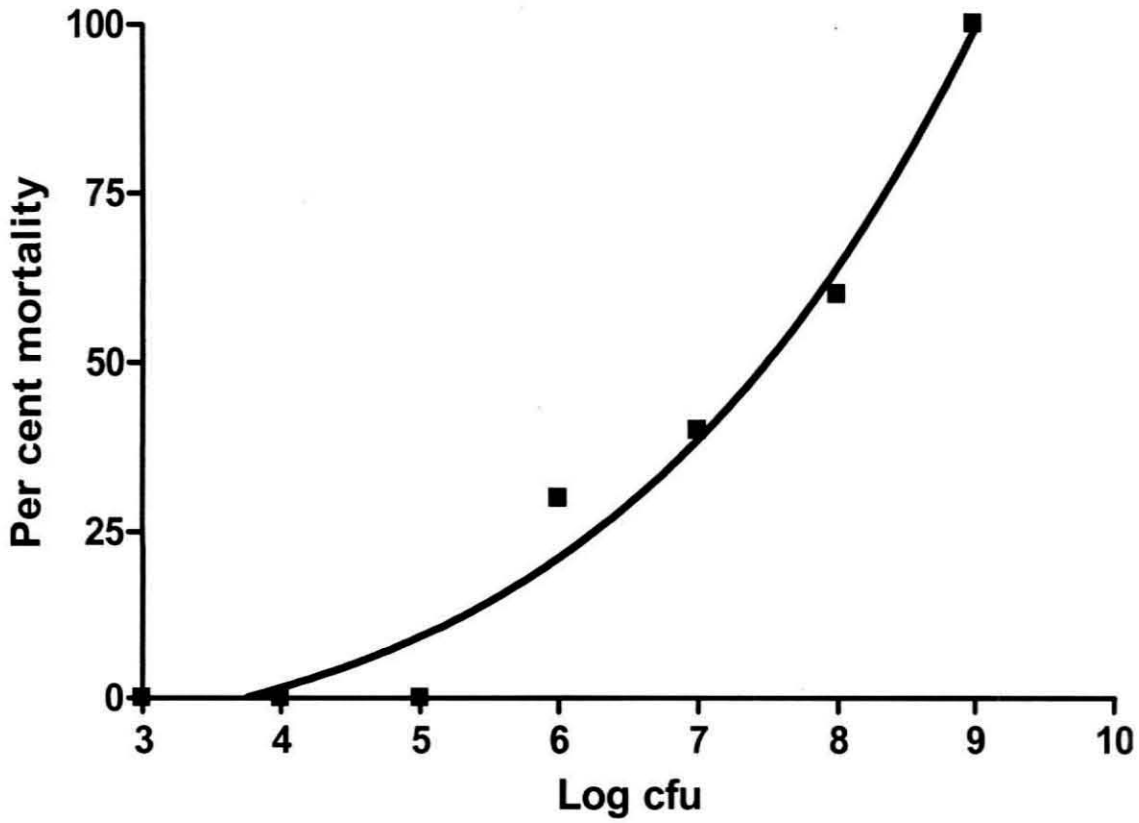


Table 12. Enumeration of *V.alginolyticus* in haemolymph of shrimps challenged with *Vibrio alginolyticus* (n=3)

Time (h) post Infection	x 10^3 cfu/ml of haemolymph (Mean±SE)		
	Shrimps fed with		
	Control	Free cells	Biofilm cells
3	0	0	0
6	1.50±0.09	1.55±0.22	1.21±0.05
12	3.82±0.09	3.56±0.04	3.04±0.31
24	5.13±0.26	3.78±0.12 a**	3.35±0.15 a***
48	5.64±0.46	4.79±0.45	1.90±0.39 a*** b***
72	7.48±0.33	1.74±0.04 a***	0.72±0.06 a*** b*
96	5.86±0.28	1.45±0.09 a***	0 a*** b***
120	3.77±0.42	1.00±0.11 a***	0 a*** b*

a = Free cell and biofilm group compared with control
b = Biofilm group compared with free cell group
* = P<0.05; ** = P<0.01; *** = P<0.001

4.3.1.4 Histopathology

Under H & E staining, colonization of bacteria along with circumscribed haemocytic accumulation as nodules forming capsules or plugs in the musculature and sinusoids was evident (Figs. 19-28). Grading of histological lesions observed in the present study in different experimental groups is shown in Table 14. Severity of the lesions as evidenced by number of haemocytic plugs in a cross section of abdominal tissue was severe in control group (grade A: 8/10), moderate in free cell treated group (grade B: 7/10) and mild in biofilm treated shrimps (grade C: 9/10). The lesions in general consisted of nodular accumulation of haemocytes around the bacteria, rounding and sloughing of hepatopancreatic cells into the tubular lumen and colonization of bacteria as basophilic amorphous plaque in abdominal musculature.

The sections stained with Gram's stain revealed extensive infiltration of haemocytes along with the presence bacteria as pink short rods was evident in the abdominal musculature (Fig.29).

4.3.1 Resistance of *P.monodon* juveniles to White Spot Syndrome Virus

4.3.1.1 Relative Per cent Survival

Relative Per cent Survival of shrimps fed with biofilm or free cells of *V.alginolyticus* and challenged with WSSV is shown in Table 13. The relative per cent survival was significantly ($P<0.001$) higher (56.46) in biofilm fed group when compared to free cell treated shrimps (18.81) at the end of the experiment (168 h).

Table 13. Relative per cent survival of shrimps fed with biofilm or free cells of *V.alginolyticus* and challenged with WSSV or *Vibrio alginolyticus*

Challenged with:	Per cent survival	
	Biofilm cell	**Free cell**
V.alginolyticus	62.10^{a}	20.10^{b}
WSSV	56.46^{a}	18.81^{b}

Values with different superscripts within the same row have significant variation ($P<0.001$)

4.3.1.2 Immunodot

The shrimps in control group infected with WSSV were positive by immunodot till the termination of the experiment while the shrimps in free cell and biofilm treated groups were WSSV positive up to 144 h and 96 h PI, respectively. The shrimps which were administered TN buffer only were WSSV negative by immunodot throughout the period of experiment.

4.3.1.3 Histopathology

Grading of histological lesions observed in WSSV infection in different experimental groups is shown in Table 15. The histological lesions were more conspicuous in the cuticular epithelium. The cuticular epithelial cells of organs like gills, haematopoietic tissue and antennal gland showed presence of intra-nuclear inclusion bodies in different stages of development under H & E staining (Fig. 30-38). In many instances, cuticular epithelium and sub cuticular connective tissue cells revealed presence of intra-nuclear inclusions which were centro-nuclear, eosinophilic and separated from the nuclear membrane with marginated chromatin by an artificial halo (Fig. 33). In advanced cases, the inclusions were basophilic, more granular in texture, and nearly filled the affected hypertrophied nucleus. In control shrimps the occurrence of inclusions in the cuticular epithelial cells was extensive and majority of the cuticular epithelial cells were occupied by the inclusions in various developing stages. The cuticular lesions as evidenced by the presence of inclusions in the hypertrophied nucleus of the cuticular epithelial cells were severe in control shrimps (Grade A: 15/15), moderate in free cell group (Grade B: 10/15) and mild in biofilm group (Grade C: 9/15). Lesions in other tissues included intra-nuclear inclusions in the hypertrophied nuclei of gill epidermal cells, tubular

epithelial cells of the antennal gland, connective tissue cells and cells of the haematopoietic tissue.

Immunoperoxidase staining of the infected cells of cuticular epithelium revealed dark brown crystalline material (Fig. 39-41) indicating positive reaction.

Table 14. Grading of histological lesions observed in shrimps infected with *V.alginolyticus* in different experimental groups

Replicates	Shrimps fed with					
	Control		Free cell		Biofilm cell	
	No. of nodules in an abdominal cross section	Grade	No. of nodules in an abdominal cross section	Grade	No. of nodules in an abdominal cross section	Grade
1	11	B	7	B	1	C
2	13	A	11	B	1	C
3	16	A	10	B	3	C
4	15	A	6	C	2	C
5	18	A	8	B	1	C
6	14	A	7	B	5	C
7	12	B	8	B	2	C
8	13	A	6	C	4	C
9	14	A	9	B	2	C
10	13	A	4	C	7	B

Table 15. Grading of histological lesions observed in shrimps infected with WSSV in different experimental groups

Replicates	Shrimps fed with					
	Control		Free cell		Biofilm cell	
	Per cent cuticular cells containing I/N inclusion bodies	Grade	per cent cuticular cells containing I/N inclusion bodies	Grade	per cent cuticular cells containing I/N inclusion bodies	Grade
1	80	A	47	B	2	C
2	72	A	49	B	3	C
3	93	A	46	B	12	B
4	88	A	48	B	8	B
5	76	A	59	A	4	C
6	84	A	38	B	5	C
7	88	A	65	A	18	B
8	75	A	47	B	22	B
9	92	A	41	B	4	C
10	96	A	46	B	3	C
11	90	A	52	A	2	C
12	89	A	56	A	7	B
13	72	A	54	A	4	C
14	79	A	47	B	16	B
15	77	A	48	B	4	C

Fig.19. Section of the abdominal muscle to show the presence of haemocytic nodules (arrow) and degenerative changes (arrow head) in the control shrimps infected with *Vibrio alginolyticus*

(H & E X 400)

Fig.20. Section of the abdominal muscle to show the presence of multiple haemocytic nodules (arrow) in the control shrimps infected *with Vibrio alginolyticus*

(H & E X 200)

Fig.21. Section of the abdominal muscle to show the presence of multiple haemocytic nodules (arrow) in the muscle tissue of free cell fed shrimps infected with *Vibrio alginolyticus*

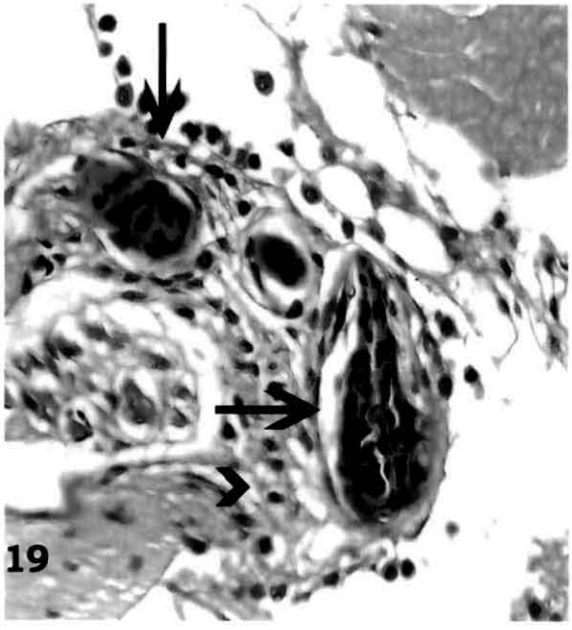
(H & E X 400)

Fig.22. Section of the abdominal muscle to show the presence of well developed haemocytic nodules (arrow) in the free cell fed shrimps infected with *Vibrio alginolyticus* (H & E X 400)

Fig.23. Muscle tissue section of biofilm fed shrimps experimentally infected with *Vibrio alginolyticus* to show a well developed haemocytic nodule (arrow) with degenerative changes (arrow head) (H & E X 400)

Fig.24. Section of the abdominal muscle to show the presence of a haemocytic nodule (arrow) in the biofilm treatment group infected with *Vibrio alginolyticus*

(H & E X 400)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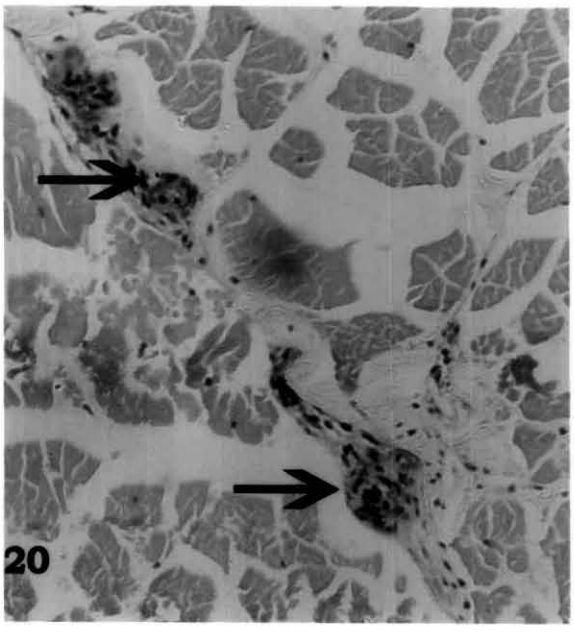
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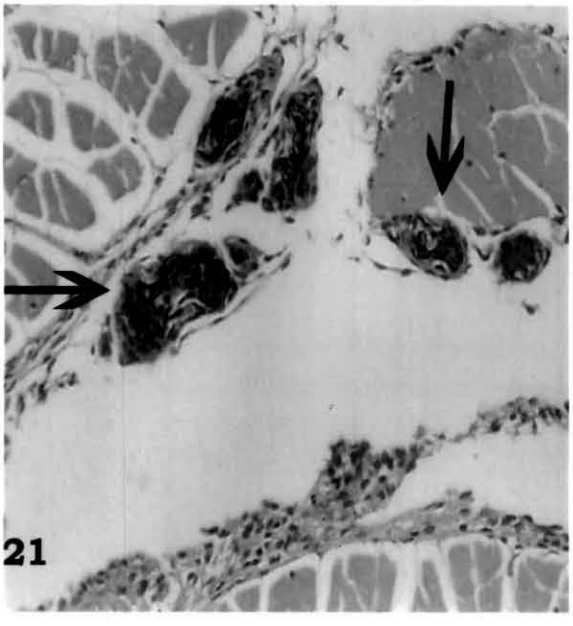
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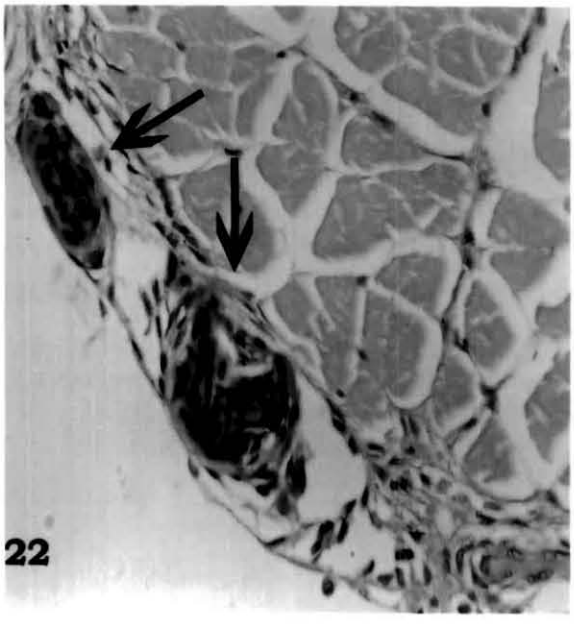
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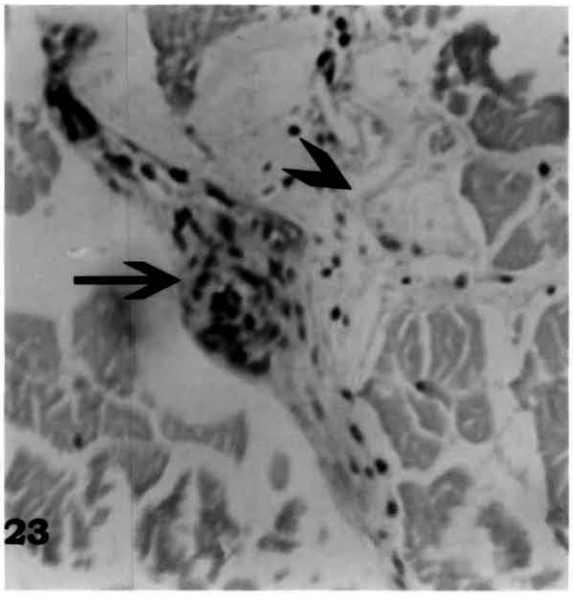
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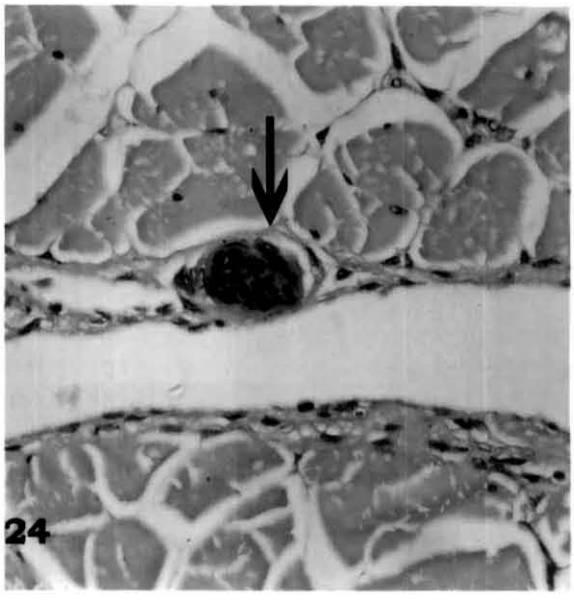
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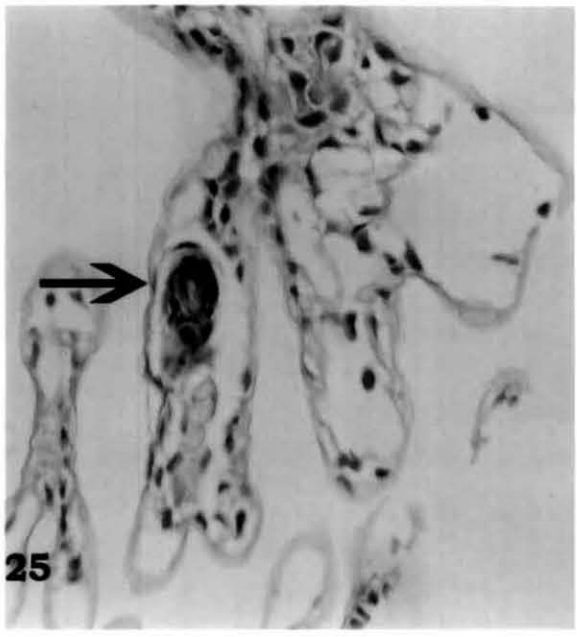
Fig.25. Section of the gill lamellae to show the presence of a haemocytic nodule (arrow) in the biofilm fed shrimps infected with *Vibrio alginolyticus* (H & E X 400)

Fig.26. Section of the abdominal muscle tissue to show the initial stage of *Vibrio* infection (arrow) with degenerative changes (arrow head) (H & E X 200)

Fig.27. Section of the abdominal muscle tissue to show the presence of a well developed haemocytic nodule (arrow) in biofilm fed shrimps experimentally infected with *Vibrio alginolyticus* (H & E X 400)

Fig.28. Section of the abdominal muscle to show the presence of a haemocytic nodule (arrow) with degenerative changes (arrow head) in the biofilm fed shrimps experimentally infected with *Vibrio alginolyticus* (H & E X 400)

Fig.29. Section of the muscle tissue to show the presence of Gram -ve bacteria (arrow) and haemocytic infiltration (arrow head) (Gram's X 200)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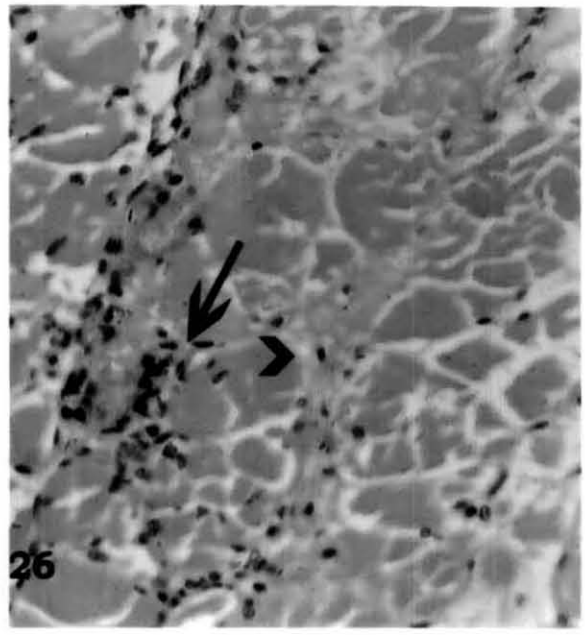
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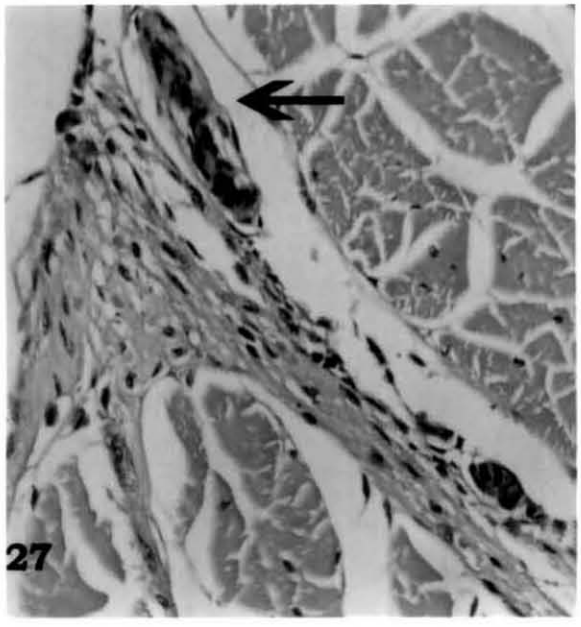
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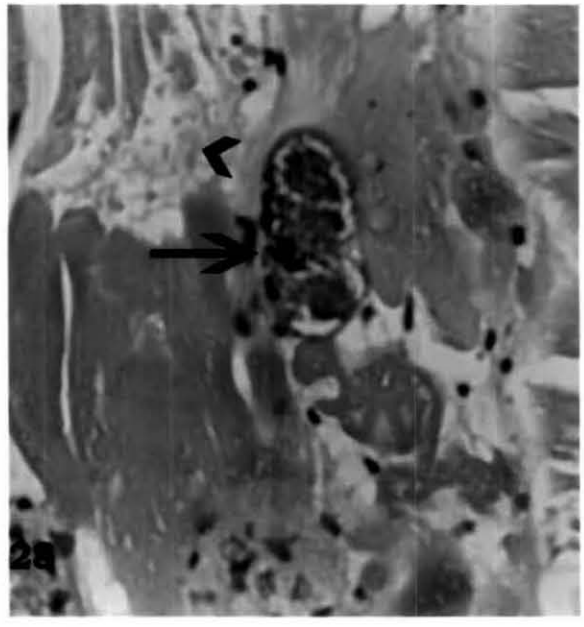
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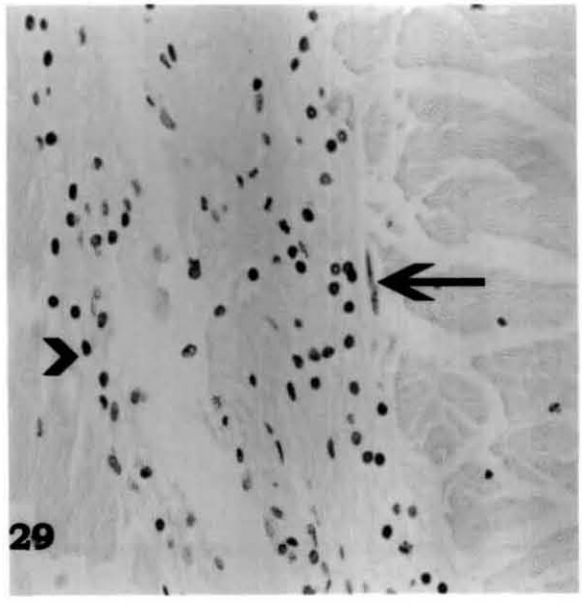
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Fig.30. Section of the cuticle to show the normal appearance of cuticular epithelium (H & E X 200)

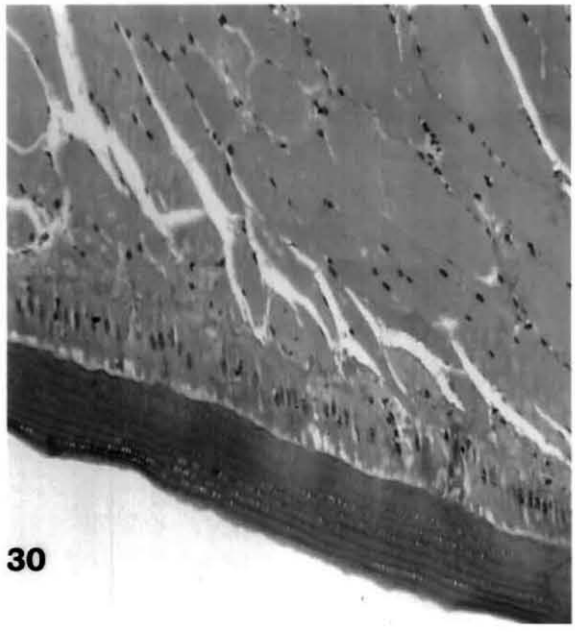
Fig.31. Section of cuticular epithelial and connective tissue cells of WSSV infected control shrimps to show the presence of basophilic intra-nuclear inclusions (arrow) (H & E X 400)

Fig.32. Section of cuticular epithelial cells of WSSV infected control shrimps to show the presence of eosinophilic intra-nuclear inclusions (arrow) (H & E X 400)

Fig.33. Section of the cuticular epithelial cells of WSSV infected control shrimps to show the presence of conspicuous eosinophilic inclusion bodies in the hypertrophied nuclei with clear halo rings (arrow) (H & E X 400)

Fig.34. Section of the cuticular epithelial cells to show the presence of basophilic intra-nuclear inclusions (arrow) in free cell fed shrimps infected with WSSV (H & E X 400)

Fig.35. Section of the cuticular epithelial cells to show the presence of conspicuous basophilic intra-nuclear inclusions (arrow) in the hypertrophied nuclei of biofilm fed shrimps infected with WSSV (H & E X 400)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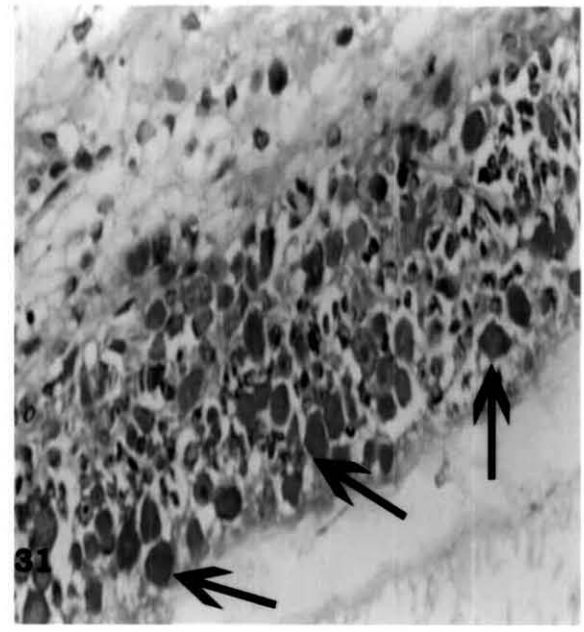
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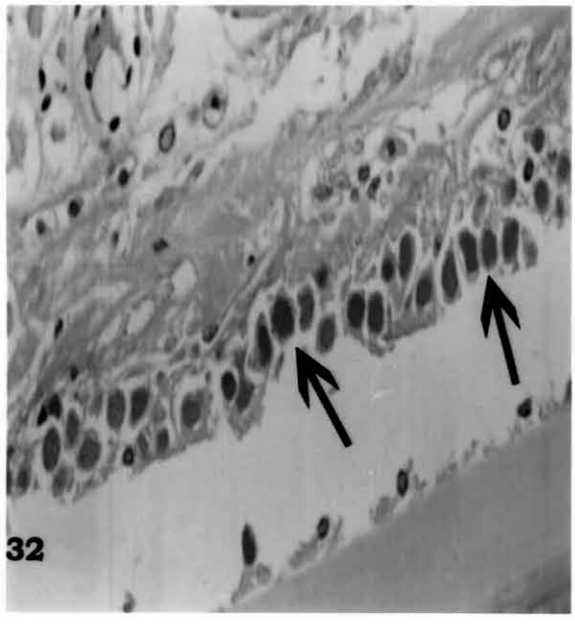
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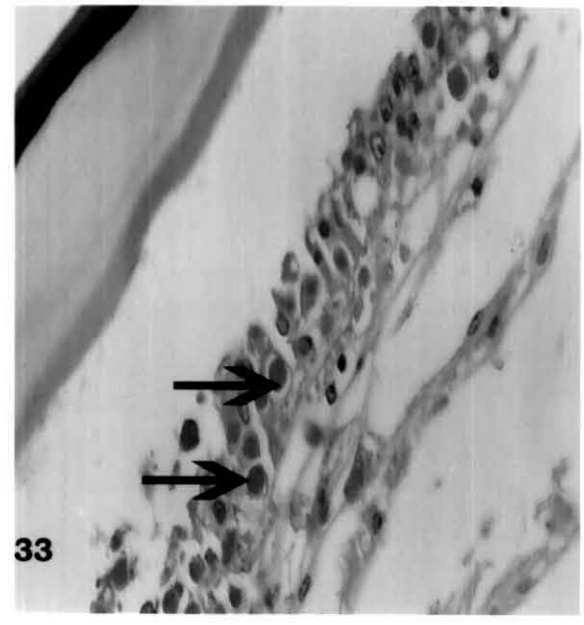
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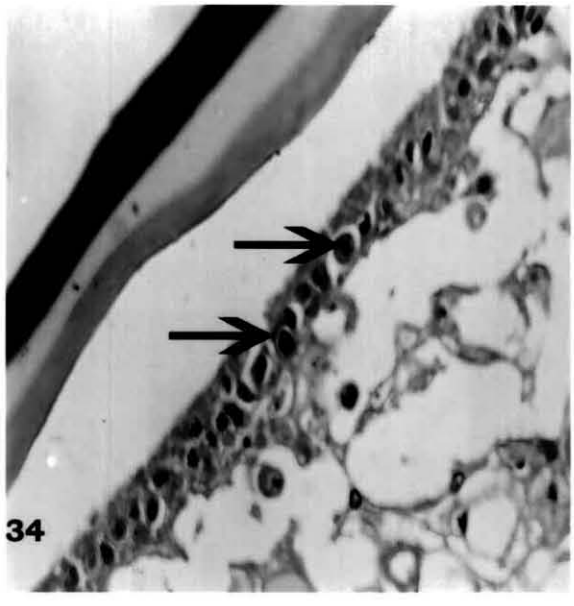
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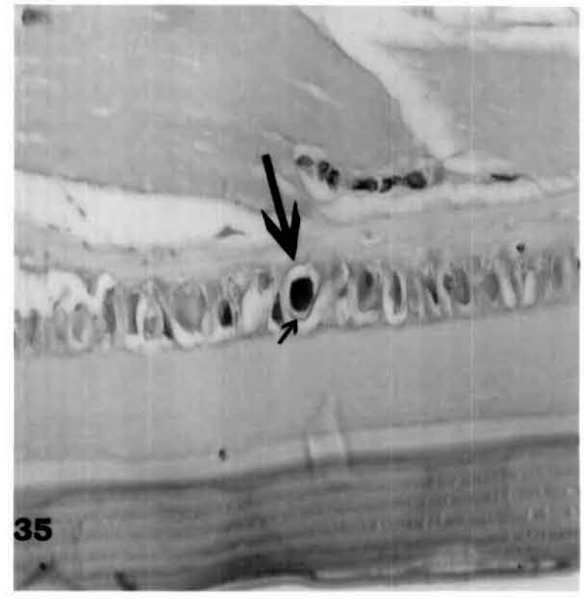
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Fig.36. Section of appendage of WSSV infected biofilm fed shrimps to show the presence of eosinophilic inclusions in the hypertrophied nuclei of cuticular epithelial cells (arrow)

(H & E X 200)

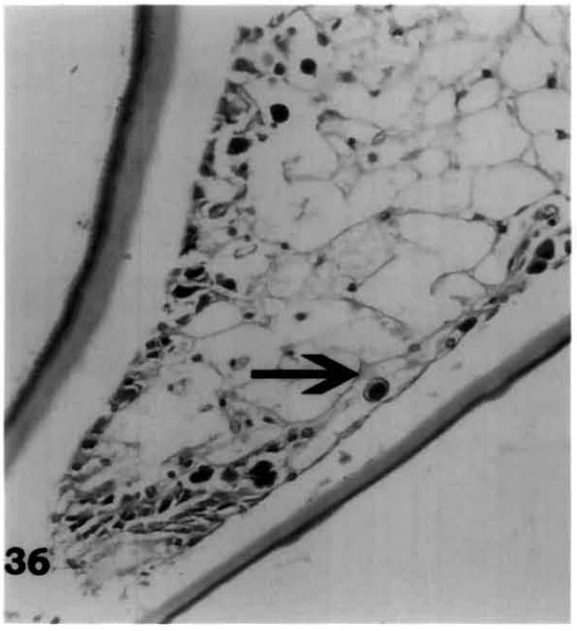
Fig.37. Section of gill epidermal cells to show basophilic inclusions (arrow) in the hypertrophied nuclei of biofilm fed shrimps infected with WSSV (H & E X 200)

Fig.38. Section of the antennal gland to show the presence of basophilic intra-nuclear inclusions (arrow) in the hypertrophied nuclei of free cell fed shrimps infected with WSSV (H & E X 400)

Fig.39. Section of cuticular epithelial and connective tissue cells of WSSV infected control shrimps to show the presence of intra-nuclear inclusions (arrow) (IP X 400)

Fig.40. Section of epithelial cells of mid gut to show the presence of intra-nuclear inclusions (arrow) in WSSV infected shrimps (IP X 200)

Fig.41. Section of appendage of biofilm fed shrimps infected with WSSV to show the presence of inclusions in the nuclei of cuticular epithelial cells (arrow) (IP X 200)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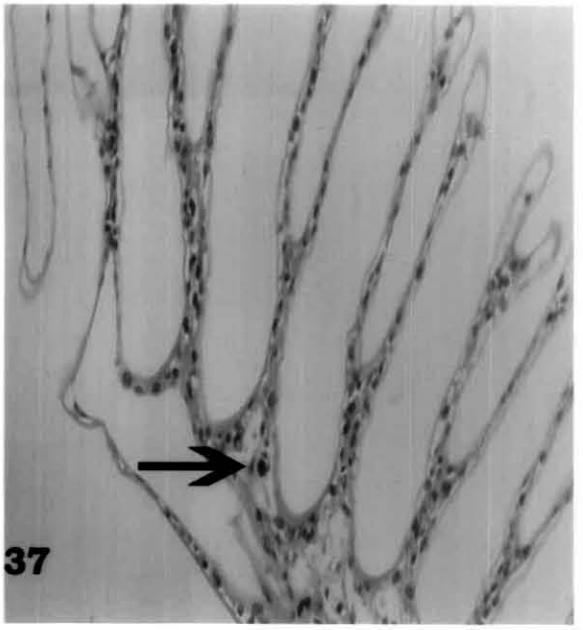
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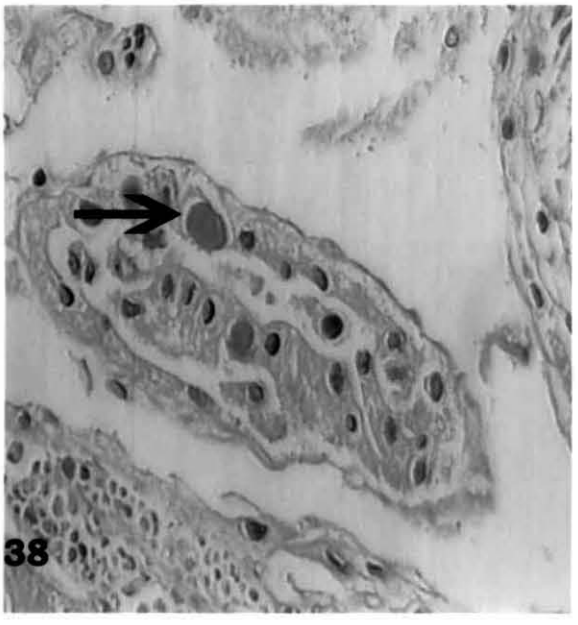
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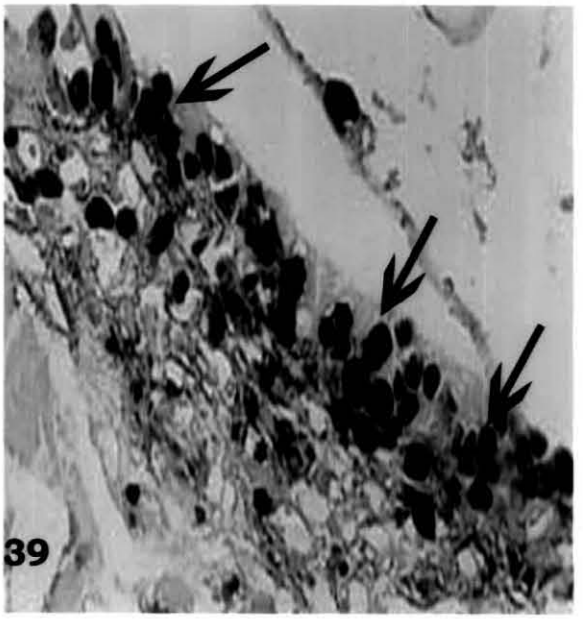
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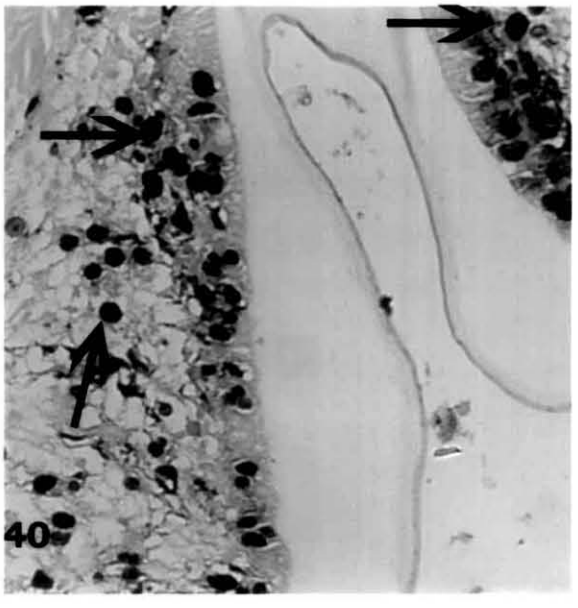
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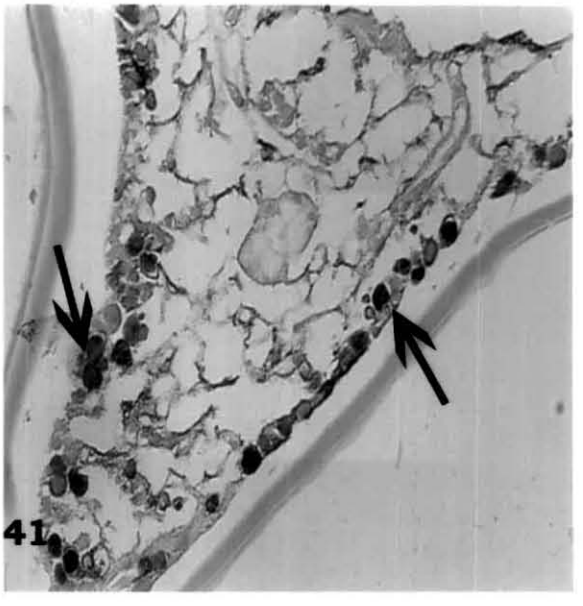
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DISCUSSION

5. DISCUSSION

The present study was undertaken to evaluate the effect of biofilm of *Vibrio alginolyticus* on the immune response and resistance against vibriosis and white spot syndrome in juvenile *Penaeus monodon*.

5.1 Development of biofilm and free cells of *Vibrio alginolyticus*

Vibrio alginolyticus, a Gram negative marine bacteria and a representative of halophilic vibrios, was isolated from a naturally infected *P. monodon* and identified based on biochemical characteristics. Besides its pathogenicity to man, this bacterium is regarded as the most important pathogen in aquaculture. The organism causes tremendous damage to crustaceans, especially in shrimps/ prawns breeding and lobstes (Brinkley *et al.*, 1976; Abraham *et al.*, 1996). Hence, this organism was selected for studying the effect of its biofilm form on immunity and pathogen resistance in the cultured shrimp, *Penaeus monodon*.

5.1.1 Nutrient requirement for optimum biofilm production

Among the six concentrations of TSB used in the present study, highest cfu of biofilm cells was obtained with 0.15 per cent TSB supplemented with 2 per cent NaCl. Umesh *et al.* (1999) used 4 different concentrations of TSB and obtained highest cfu of biofilm cells of *Aeromonos hydrophila* with 0.225 per cent. In general, biofilm formed was better at lower concentration of the medium. As the growth of *V.alginolyticus* was inhibited when salt was not supplemented (Selvin and Lipton, 2003), a uniform final concentration of 2.5 per cent NaCl was provided in the present study.

5.1.2 Growth kinetics of biofilm and free cells of *Vibrio alginolyticus*

In the present study, density of biofilm cells increased gradually, reaching a peak by Day 3 of incubation (4.47 x 10^{11}/g chitin flakes). On the other hand, the planktonic cell density reached its peak on Day 2 of incubation (2.70 x 10^{12}/ml) and decreased afterwards corresponding to an upward trend in biofilm cell population. Increased colonization with time resulted in an increase in biofilm cell population with a corresponding decrease in the population of planktonic cells. Similar observations were reported by Anwar *et al.* (1992) and Azad *et al.* (1999) in *Staphylococcus aureus* and *A.hydrophila*, respectively. In the former, planktonic cell population of *S. aureus* remained steady at 5 x 10^9 cfu/ml after 24 h of inoculation, while the number of biofilm cells increased from 2 x 10^5 cells/ cm to 8 x 10^7 cells/cm of silicon tubing. In the later, the planktonic cell population of *A.hydrophila* reached its peak of 2.13 x 10^8 cells on Day 2 and started declining with a cell population of 1.03 x 10^5/ml on Day 5, while the biofilm cell density reached its peak at 4.6 x 10^{11}/g chitin flakes on Day 4 and then started declining. The present study indicated that the biofilm formation in conducive environment will gradually increase with time irrespective of bacterial species.

5.1.3 Inactivation of *V.alginolyticus* biofilm and free cells

Biofilm cells of *V. alginolyticus* grown on chitin flakes were subjected to varying degree of heat and concentration of formalin. The 3-day-old biofilm cells were completely inactivated at 80°C in 10 min and 10 per cent formalin in 24 h. In contrast, free cells of *V. alginolyticus* were inactivated at 60°C in 10 min and 0.5 per cent formalin in 24 h. Azad *et al.* (1999) were able to completely inactivate biofilm cells of *A.hydrophila* at 90°C in 30 min. The present study revealed that the biofilm cells of *V.alginolyticus* were more resistant to heat and chemical

treatment compared to planktonic cells. This could be due to aged biofilm cells embedded in multilayered glycocalyx, which rendered protection from heat and chemical action. In the present study, heat inactivated biofilm cells were used as oral immunostimulant through artificial feed. It was found that heat inactivated biofilm cells elicited higher protective response than formalin inactivated bacterin as heat inactivated cells exposed large number of immunogenic epitopes (Lamers and Muiswinkel, 1986).

5.1.4 Protein profile of biofilm and free cells of *V.alginolyticus*

SDS-PAGE profile of *V. alginolyticus* free cells revealed 22 protein bands, while that of biofilm cells revealed a repression of four proteins of molecular weight 31, 44, 48, and 53 kDa corresponding to free cells and additional expression of three proteins of 45, 50, and 55 kDa. Asha *et al.* (2004) reported that the biofilm cells of *A.hydrophila* showed repression of 9 proteins and additional expression of 3 proteins of 72, 36 and 22 kDa. Similar observations were also made in *Pseudomonas putida* where 15 proteins were up-regulated and 30 proteins were down-regulated following attachment to a silicon surface (Sauer and Camper, 2001). Another related study under iron limited conditions has shown that *P.aeruginosa* expressed additional 77 and 85 kDa proteins (Anwar *et al.*, 1984). Hodgson *et al.* (1995) have reported repression of a 48 kDa protein in the biofilm cells of *P.aeruginosa.* Biofilm of *P.aeruginosa* developed on glass wool also revealed changes in the protein profile (Steym *et al.*, 2001). Studies on starved cells of *A.hydrophila* have shown that about five outer membrane proteins were lost with the expression of three new proteins compared to free cells (Rahman *et al.*, 1998). Also, starved cells of *Vibrio* spp. lost many cellular proteins and synthesized new proteins called starvation proteins (Amy and Morita, 1983; Nelson *et al.*, 1997). The variation in protein profile of biofilm cells compared to planktonic cells in the present study and earlier studies might be due to

their physiological and phenotypical differences. Also, expression of additional proteins in biofilm cells could be a stress response (Buchmeier and Hefron, 1990) which might help the bacteria to survive in the new mode of life. Bacteria infecting tissues produce cell surface components not found on the bacteria grown *in vitro* (Brown and Williams, 1985) and the cell surface changes may be produced in the cells of the same species in response to variations in nutrient status, surface growth and other environmental factors (Lorian *et al.*, 1985).

5.2 Standardization of dose of biofilm cells for use as immunostimulant in juvenile *Penaeus monodon*

5.2.1 Immune response in shrimp exposed to different biofilm doses

In the present study, the bacterial biofilm cells were administered to shrimps through feed. Immunostimulants can be administered to marine animals through oral (Itami *et al.*, 1994; Takahashi *et al.*, 2000), immersion (Sung *et al.*, 1994) and direct injection (Smith and Soderhall, 1983) methods. But immersion and injection methods may induce severe stress on shrimps. Therefore, oral administration was found to be a useful delivery system to large number of shrimps at any stage of life (Itami *et al.*, 1994).

In the present study, the values of various immune parameters in biofilm treated shrimps were higher than those observed in control shrimps. Values of immunological parameters were highest in 10^9 and 10^{10} cfu of biofilm treated shrimps and there was no significant difference between them. In addition, the values were higher on 14th day post feeding than that on Day 7. This could be due to the fact that the shrimp needed some time to adapt or respond immunologically to their changing environment (Sung *et al.*, 1994). There was no significant difference between the groups fed with 10^9 and 10^{10} cfu biofilm cells among the various immune parameters studied. These findings clearly

showed that the immune system of the shrimp could be activated by feeding biofilm cells of *Vibrio alginolyticus*. The optimum dose of biofilm cells of *V.alginolyticus* as an immunostimulant to activate the immune system of juvenile *P. monodon* was found to be 10^9 cfu/g shrimp. Boonyaratpalin *et al.* (1993) documented that the growth and immune response of tiger shrimp were inhibited by extra dosages of peptidoglycan by oral administration. Therefore, it can be construed that the feeding period and dosage of immunostimulants are vital for a positive immune response.

Haemocyte counts were significantly higher in all the treatment groups in the present study whereas in 10^9 and 10^{10} cfu biofilm fed groups, the counts were highest when compared to 10^8 cfu and control shrimps. Also, at 10^8 cfu, the counts were not significantly different between Day 7 and 14. The pattern of haemocytosis on Day 7 and 14 in 10^9 and 10^{10} cfu groups were also similar. These results indicated that 10^8 cfu of biofilm cells were not sufficient to activate haemocyte synthesis as compared to higher doses. However, it is very imperative to have a thorough understanding of defense mechanism and haemocyte function in crustaceans (Roch, 1999). Further, crustacean haemocytes are thought to be analogous to vertebrate leucocytes and play an important role in removing foreign particles such as bacteria from haemolymph by phagocytosis (Ratner and Vinson, 1983). Le Moullac *et al* (1998) reported that the enumeration of THC was a potential indicator of immune status in crustaceans. In crustaceans, THC is regarded as a stress indicator, but varies non-specifically according to the nature of rhythms of the environment, moulting, development of organs, reproductive status, nutritional conditions, diseases, as well as chemical and physical stresses (Campa-Courdova *et al.*, 2002b). A lower–than-normal number of circulating haemocytes in crustaceans correlate well with a reduced resistance to pathogens (Le Moullac *et al.*, 1998; Le

Moullac and Haffner, 2000). In the light of the above reports, it could be opined that haemocytosis in 10^9 and 10^{10} cfu groups in the present study was a consequence to immunostimulation by biofilm cells.

Increased haemocyte counts were also reported when shrimps were administered various immunostimulants like probiont bacterium (Rengpipat *et al.*, 2000), β 1,3 glucan (Lopez *et al.*, 2003), vitamin C (Lee and Shiau, 2003), spent brewer's yeast β-glucan (Suphantharika *et al.*, 2003), vitamin E (Lee and Shiau, 2004), sodium alginate (Cheng *et al.*, 2005b), chitin/ chitosan (Wang and Chen, 2005), hot water extracts of *Gracilaria* spp. (Hou and Chen, 2005) and *Gelidium* spp. (Fu *et al.*, 2006), *Sargassum fusiforme* polysaccharide extracts (Huang *et al.*, 2006), marine yeast (Sajeevan *et al.*, 2006), *Dunaliella* extract (Supamattaya *et al.*, 2005) and certain Indian immunostimulant herbs (Citarasu *et al.*, 2006). The increased haemocyte count in biofilm fed shrimps in the present study is in conformity with the above research findings.

Phenoloxidase activity was conspicuously elevated in 10^9 and 10^{10} cfu groups than in 10^8 cfu group. Unlike THC values, significant difference was found between the values observed on Day 7 and 14 in 10^8 cfu group. Also, the values in 10^9 and 10^{10} cfu group did not differ significantly either on Day 7 or on 14.

Phenoloxidase (PO), the key enzyme in the synthesis of melanin, occurs in haemocytes as an inactive pro-enzyme, prophenoloxidase (proPO). proPO is activated to form PO when it reacts with a zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, or heat (Sritunyalucksana and Soderhall, 2000). Direkbusarakom *et al.* (1996) reported that apart from their role in melanization, components of the putative proPO activity system stimulated several cellular defense reactions, including phagocytosis, nodule formation, encapsulation, and haemocyte migration. PO activity

could be enhanced *in vitro* by β-glucan treatment which may contribute to increased disease resistance in tiger shrimp (Sung *et al.*, 1994). Sung *et al.* (1996) also confirmed that PO activity could be enhanced by immunostimulants (heat-killed *Vibrio* antigen, β-1, 3-1, 6-glucan and zymosan). Activation of proPO system which is measured in terms of PO activity has been used by some investigators to measure immunostimulation in shrimps (Sung *et al.*, 1994; Devaraja *et al.*, 1998). *Penaeus japonicus*, which had been fed a diet containing LPS extracted from *P.agglomerans* at dose of 20 μg/Kg shrimp for 7 days had increased PO activity (Chang *et al.*, 2003). In *P.monodon* which had been fed a diet containing β-1, 3-glucan at 2, 10 or 20 g/ Kg for 20 days and challenged with WSSV, PO activity decreased immediately after challenge but returned to normal after 3 days and attained the highest level after 9 days (Takahashi *et al.*, 2000). Also, PO activity increased significantly in shrimp fed dietary sodium alginate (Cheng *et al.*, 2005b). Other studies have also reported elevated PO activity after exposing the shrimp to immunostimulants like probiont bacterium (Rengpipat *et al.*, 2000), β 1,3 glucan (Lopez *et al.*, 2003), vitamin C (Lee and Shiau, 2003), spent brewer's yeast β glucan (Suphantharika *et al.*, 2003), vitamin E (Lee and Shiau, 2004), hot water extracts of *Gracilaria* spp. (Hou and Chen, 2005), *Dunaliella* extract (Supamattaya *et al.*, 2005), chitin/ chitosan (Wang and Chen, 2006),) and *Gelidium* spp. (Fu *et al.*, 2006), *Sargassum fusiforme* polysaccharide extracts (Huang *et al.*, 2006), marine yeast (Sajeevan *et al.*, 2006) and certain Indian immunostimulant herbs (Citarasu *et al.*, 2006). The results of the present study agree with the previous findings. Further, it is construed that the increased PO activity in biofilm treated shrimps in the present study could be attributed to increase in the number of circulating haemocytes. Alternatively, the increased PO activity may also be due to direct activation of proPO enzyme. Sung *et*

al. (1996) suggested that the stimulative mechanism in the proPO activating system may differ according to the immunostimulant used.

Total protein content was rather inconsistent in the biofilm treated individuals when compared to other immune parameters in the present study. 10^8 cfu biofilm cells in the present study had no effect on the protein content. Also, in 10^9 and 10^{10} cfu groups, the protein content increased only on Day 14. Further, the values were not significantly different between the groups exposed to 10^9 and 10^{10} cfu of biofilm cells. Haemocyanin represents 80-95 per cent of total protein in the haemolymph of crustaceans (Djangmah, 1970). Haemocyanin is affected by the moulting cycle, nutritional conditions and stress factors (Boone and Schoffenich, 1979; Hagerman, 1983).

The increased total haemolymph protein content in biofilm treated shrimps in the present study might be due to the stimulation of protein synthesis sites such as the digestive gland where haemocyanin is synthesized. The shrimp haemocytes are synthesized in the haemopoietic tissue located in the paired epigastric nodules just over the digestive gland where haemocyanin is synthesized. Hence, when biofilm cells activate the haemopoietic tissue, they may also stimulate the haemocyanin synthesis site (Gellisen *et al.*, 1991).

Antibacterial activity in terms of Survival Index was more pronounced in 10^9 and 10^{10} cfu groups when compared to 10^8 cfu and control shrimps. Also, the immune response was more conspicuous on Day 14 than on Day 7. There was no significant difference in antibacterial activity between Day zero and Day 7 in 10^8 cfu group suggesting that shrimps are less responsive to biofilm cells at this dose with respect to antibacterial activity within 7 days of exposure.

Sung *et al.* (1996) were able to enhance the bactericidal activity by feeding immunostimulants to tiger shrimp. Invertebrates, owing to their open type circulation are particularly vulnerable to bacterial invasion through wounds or abrasions from their microbe rich habitat (Smith *et al.*, 1995b). Antibacterial activity could be elicited when shrimps are exposed to bacteria. Substances that have attracted the research interest, especially in shore crabs and lobsters are bactericidins (Cornick and Stewart, 1975). However, these substances were not found in the plasma (Smith and Ratcliffe, 1978), but were present in the haemocytes (Chisholm and Smith, 1991). Sung *et al.* (1996) reported the presence of antibacterial substances in the plasma of tiger shrimp and suggested that this could be released from haemocytes by exposing the shrimp to immunostimulants. Sung *et al.* (1996) reported that the bactericidal response exhibited by immunostimulants in shrimp was rapid and short lived. The increased survival index value in biofilm treated shrimps in the present study highlighted the importance of antibacterial proteins in the haemocytes of shrimp in providing better immunity.

5.2.2 Comparative studies on the immune functions of shrimp exposed to biofilm and free cells of *Vibrio alginolyticus*

Mean values of total haemocyte count, phenoloxidase activity, total hemolymph protein and antibacterial activity were significantly higher in 10^9 cfu biofilm treated shrimps than free cell treated groups. The comparison of these immunological parameters in control, free cell and biofilm group clearly showed that biofilm cells were superior in activating the immune system than the free cells. This might be due to the protection afforded by the glycocalyx of biofilm. The glycocalyx being a polymer of neutral hexoses (Costerton *et al.*, 1981) probably acts similar to a microcapsule in protecting the antigen against degradation in the gut as observed in encapsulated antigen microspheres (Dalmo *et al.*, 1995; Polk *et al.*, 1994). Enhanced uptake of antigens was possible

through anal intubation, bypassing the foregut environment leading to stimulation of high systemic antibody titres (Rombout *et al.*, 1986) and protective responses (Agius *et al.*, 1983). Azad *et al.* (2000) also demonstrated better protective responses in common carp administered biofilm cells of *A.hydrophila* by oral route. Since biofilm cells are embedded in the glycocalyx matrix, there is slow and sustained release of antigens for a continued immunological activation when compared to free cell antigens that are rapidly released making the immune activation short lived and insufficient. The increased immunological values in the biofilm fed group in the present study perhaps substantiate the above observations.

5.3 Resistance of *P.monodon* juveniles exposed to biofilm and free cells of *Vibrio alginolyticus*

5.3.1 Resistance of *P.monodon* juveniles to *Vibrio alginolyticus*

LD_{50} study confirmed that the *Vibrio alginolyticus* isolate was virulent to shrimp and the LD_{50} (24 h) value was found to be 3.12×10^7 cfu/shrimp (1.7±0.4 g). Selvin and Lipton (2003) reported that the LD_{50} (24 h) of *V.alginolyticus* to *P.monodon* (40 DOC, 2.6±0.52 g) was 5×10^6 cfu/ shrimp. In the present study, LD_{50} was found to be higher than the above.

Protective response of biofilm and free cells of *V. alginolyticus* was evaluated by challenge studies. The Relative Per cent Survival was significantly higher in biofilm fed shrimp when compared to free cell fed shrimps. In experimental vibriosis, the histological lesions of hemocytic capsules surrounding the bacterial colonization were severe in control shrimps which were not fed either biofilm or free cells. The lesions were moderate in free cell fed shrimp and mild in biofilm fed shrimps. In the present study, *V.alginolyticus* was completely cleared from the haemolymph of biofilm cell treated group at 72 h PI and free cell treated

group at 96 h PI while the haemolymph of control shrimps showed presence of bacteria even at 120 h PI. These results clearly indicated that the biofilm fed shrimps were more efficient in clearing the bacteria from haemolymph when compared to free cell fed shrimps or control shrimps. The histological lesions observed in different treatment groups appeared well correlated to RPS and enumeration of *V.alginolyticus* in the haemolymph.

Increased protection against vibriosis caused by *V.alginolyticus* and *V.harveyi* was observed in shrimps given immunostimulants like sodium alginate Cheng *et al.*, 2005b), *Sagassum fusiforme* polysaccharide extracts (Huang *et al.*, 2006) or probiont bacterium (Rengpipat *et al.*, 2000). Teunissen *et al.* (1998) found that vaccination of *P.monodon* with polyvalent vaccine prototypes could significantly enhance the resistance of shrimp to vibriosis. Effective vaccination of *P.monodon* and *P. japonicus* against vibriosis with a formalin killed *Vibrio* spp. vaccine was reported by Kou *et al.* (1989) and Itami *et al.* (1989) respectively. They also found an effective protection against vibriosis through the use of β-1, 3-glucan as an immunostimulant. Itami *et al.* (1989) reported that the prawns injected or immersed with formalin-killed *Vibrio* bacterin experienced reduced mortality when they were challenged with *Vibrio* injection 30 days later. Oral administration of Schizophyllan, a β-1, 3-glucan extracted from the fungus *Saccharomyces commune* has been reported to increase the resistance of *M. japonicus* against *Vibrio* spp. (Itami, *et al.*, 1994). In the present study, bacterial biofilm cells were able to improve the survival of shrimp infected with pathogenic *V.alginolyticus*.

Vibrio spp., a principal pathogen of shrimp, is known to cause shell disease, localized infection and bacterial septicemia (Lewis, 1973; Lightner and Lewis, 1975; Sindermann, 1977; Liao *et al.*, 1985; Liu and

Chen, 1988). Since 1986, there has been numerous occurrence of massive mortality in cultured tiger shrimp worldwide and researchers have found that 80 per cent of the bacteria isolated from these instances were *Vibrio* spp. (Liu and Chen, 1988; Song *et al.*, 1993). The increased incidence of vibriosis in cultured shrimps coupled with an awareness of the problems associated with use of antibiotics has led researchers more towards prophylaxis.

Glucan administration seemed to have an immunostimulatory effect upto 18 days post treatment (Sung *et al.*, 1994). However, the authors suggested that as the growth cycle of penaeid shrimps is usually over 4 months, administration of glucan will only be effective if repeatedly applied. In the present study, juvenile shrimps were exposed to biofilm cells upto 14 days and then challenged with *vibrio* spp., subsequently a protective response was noticed. As the protective response is short-lived, repeated treatment with biofilm may be useful for sustained immune response and continuous exposure to immunostimalant for longer period may lead to immune exhaustion (Hauton, *et al.*, 2007). Sung and Song (1996) suggested that *Vibrio* antigen delivered by immersion can be absorbed by shrimp either through the circulatory or digestive system. They also suggested that the bactericidal reactions activated by *Vibrio* antigen treatment are short-term effects and the antigens are absorbed by shrimp via the digestive system which may contribute to disease resistance in shrimp. According to these authors, the short-lived nature of this phenomenon could be attributed to persistence of antigen for a short period. To prolong the duration of the enhanced resistance, the authors proposed that the shrimps be fed periodically with feedstuff containing *Vibrio* antigen or other immunostimulants. Protection by *Vibrio* biofilm cells against viobriosis in the present study endorsed the above findings since the biofilm cells were administered orally through the feed.

Live, culturable bacteria injected into penaeid shrimp were rapidly removed from the haemolymph, as measured by the recovery of bacterial cfu (Martin *et al.*, 1993). Martin *et al.* (1998) proposed that the gills, which have not been shown to contain fixed phagocytes, act as a passive filter, trapping haemocyte- bacterial nodules when they reach sufficient size to become lodged in the small bronchial blood vessels. In contrast to the above results, van de Brack *et al.*, (2002) reported that the live pathogenic bacterium, *V.anguillarum*, does not accumulate in the gills of *P.monodon* and opined that the lymphoid organ was the major site of bacterial uptake from the circulation. However, in the present study haemocytic nodules were not seen in the lymphoid organ but occasionally seen in gills and frequently in muscles since the bacteria were administered intramuscularly in the second segment. It is likely that the relative contribution of different tissues to the removal of bacteria from the circulation differs among species, also depends on the strain, dose, and route of entry of the pathogen (Burgents *et al.*, 2005). These authors also proposed hepatopancreas as an important site of bacterial accumulation.

The open circulatory system of crustaceans necessitates localization of immune reactivity to avoid self damage. This was achieved by sequestration of foreign antigens into haemocyte nodules or capsules and compartmentalization of the bioactive defense molecules within the blood cells (Smith *et al.*, 2003). The above findings also support the microscopic observations of experimental vibriosis reported in the present study.

5.3.2 Resistance of *P.monodon* juveniles to White Spot Syndrome Virus

Protective response in shrimp fed with biofilm and free cells of *V.alginolyticus* against WSSV was studied. The Relative Per cent Survival

was significantly higher in biofilm treated shrimps when compared to that of free cells. Histological lesions in WSSV infection consisted of intra-nuclear inclusions in various developmental stages. They were extensive in the cells of cuticular epithelium of control shrimps which were not fed either with biofilm or free cells. These inclusions were confirmed as WSSV inclusions by immunoperoxidase staining. The lesions were moderate and mild in free cell and biofilm fed groups, respectively. Also, the shrimps were negative to WSSV by monoclonal antibody based immunodot after 96 h PI in biofilm fed group and after 144 h PI in free cell fed group while the shrimps in control group which were not exposed either to biofilm cells or free cells were positive to WSSV up to the termination of the experiment. The absence of positive reaction by immunodot at 96 h post infection indicated the protective response exhibited by the biofilm cells. The histological changes could also be correlated with the severity observed based on RPS and immunodot.

Increased RPS in shrimps treated with biofilm cells and challenged with WSSV appears to have significant application in shrimp culture system where WSSV infection is the most serious disease condition that affects the economy of shrimp farming (Flegel, 2006). White spot syndrome has assumed an epidemic proportion in Southeast Asia and caused considerable economic losses (Itami *et al.*, 1998; Wang *et al.*, 1995). There are no effective antiviral drugs against WSSV available for use in aquaculture. Therefore, prophylaxis is of paramount importance for controlling viral diseases. Protection of shrimps from viral infections by feeding immunopotentiators will greatly benefit the shrimp industry (Itami *et al.*, 1998).

Increased protection against white spot disease was observed in shrimps given immunostimulants like marine yeast (*Candida sake*) and *Dunaliella* extract (Sajeevan *et al.*, 2006; Supamattaya *et al.*, 2005). Dietary administration of peptidoglycon extracted from *Bifidobacterium thermophilum* has been reported to increase the resistance of *Marsupenaeus japonicus* against *Vibrio penaeicida* and WSSV (Itami *et al.*, 1998). In an experiment on *M. japonicus*, dietary administration of LPS extracted from *Pantoea agglomerans* at 20, 40 and 100 µg/ Kg shrimp body weight for 10 days significantly increased the resistance against Penaeid rod shaped DNA virus (Takahashi *et al.*, 2000). Citarasu *et al.* (2006) observed that selected immunostimulant Indian herbs could provide better protection against WSSV in *P.monodon* and also helped to reduce the viral load of the infected shrimp. Namikoshi *et al.* (2004) studied the efficacy of vaccines made of inactivated WSSV recombinant proteins (rVP26, rVP28) with or without immunostimulants (β-1,3-glucan or killed *Vibrio penaeicida*) by intramuscular vaccination followed by intramuscular challenge of kuruma shrimp with WSSV. The results indicated that these vaccines could protect the shrimp from the virus. The results of the present study highlighted the importance of *Vibrio* biofilm cells in reducing the mortality in WSSV infected shrimps.

Immunostimulants are known to increase disease resistance against pathogens by stimulating non-specific defense mechanism. Therefore, there is no memory component involved and the response is of short duration. Use of immunostimulants is an effective means of increasing the immunocompetency and disease resistance (Sakai, 1999). Research on shrimp immunostimulants has attracted the attention of scientists world over and currently many agents are in use in shrimp aquaculture industry (Smith *et al.*, 2003).

In the present study, the bacterial biofilm cells have helped to increase the survivability of shrimps by improving the haemolymph

immune parameters. These observations and reports on earlier studies on use of various immunostimulants in shrimp culture have clearly shown that immunostimulants can activate the immune system of invertebrates and protect against infections.

Umesh *et al.*, (1999) reported that the biofilm, apart from enhancing food for fish food organisms and fish, improved water quality by lowering ammonia. The presence of biofilm reduces the necessity of water exchange, which certainly decreases the cost of shrimp production (Thompson *et al.*, 2002). Huang *et al.* (2006) reported that appropriately enhanced immune activity is an advantage for the animal's ability to resist pathogen and for growth when the animal is infected. But in a good, pathogen free environment, the over active immune response may require extra energy for no good cause which could otherwise be used for growth. From this point of view, the authors further suggested that administration of immunostimulant is not always acceptable.

The immune system encounters the actual surface components of the bacteria presented to it. Batch cultures of bacteria grown in nutrient rich media do not present the surface components which express under the biofilm mode of growth (Azad, 1997). Studies on bacterial biofilm with relevance to shrimp are lacking. However, literature available on the role of biofilm glycocalyx in mammalian and finfish health could be used for drawing comparisons. Infections with *Haemophilus influenza* type-B were found to elicit antibodies against the capsular glycocalyx of the pathogen (Mpairwe, 1971). Infections with enteropathogenic *E.coli* also elicited antibody response to both the glycocalyx and the pili of these organisms (Meyers, 1978). Another study on the immune response and protection induced by an extracellular polysaccharide having a similar composition as the capsular polysaccharide of *Aeromonas salmonocida*

(Bricknell *et al.*, 1997) also highlighted the role of glycocalyx surface layer in protection. Vaccines directed against the surface components of these pathogens were protective. Better protective response in terms of elevated antibody titre was obtained when common carps were vaccinated orally with biofilms of *Aeromonas hydrophila* (Azad *et al.*, 1999).

SUMMARY

6. SUMMARY

The present study was undertaken to develop and evaluate the biofilm of *Vibrio alginolyticus* for oral immunostimulation in tiger shrimp, *Penaeus monodon*.

Development of biofilm of *V. alginolyticus* was standardized and maximum biofilm formation was achieved with 0.15 per cent TSB supplemented with 2 per cent NaCl on 0.3 per cent chitin flakes with incubation for 3 d. The biofilm cell population was inversely related to the planktonic cell population during the culture. The biofilm cells could be inactivated at 80°C and 10 per cent formalin while free cells at 60°C and 0.5 per cent formalin suggesting the resistant nature of biofilm to action by heat and chemicals.

In SDS-PAGE, biofilm cells showed an increased expression of 3 new proteins and repression of 4 proteins compared to free cells.

Elevated values of the immune parameters with respect to total haemocyte count, phenoloxidase activity, total haemolymph protein and antibacterial activity were observed in shrimps exposed to 10^8, 10^9 and 10^{10} cfu/g shrimp/ day biofilm cells when compared to control shrimps. The values were highest in 10^9 and 10^{10} cfu groups with no significant difference between them. It was observed that 10^9 cfu/ g shrimp/day for 14 days was the optimum dose and time required to activate the immune system of juvenile *P.monodon*.

The values of immune parameters with respect to total haemocyte count, phenoloxidase activity, total haemolymph protein and antibacterial activity in shrimps fed with 10^9 cfu/g shrimp/day of biofilm cells for 14 days were significantly higher than the shrimps fed with 10^9

cfu/g shrimp/day of free cells suggesting that the biofilm cells are superior to free cells in activating the immune system of *P. monodon.*

LD_{50} (24 h) of *V.alginolyticus* to juvenile *P.monodon* was found to be 3.12×10^7 cfu/shrimp.

Highest Relative Per cent Survival was observed in shrimps exposed to 10^9 cfu biofilm cells/g shrimp/ day for 14 d and then challenged with WSSV or pathogenic *V.alginolyticus.* The biofilm fed shrimp were negative to WSSV as confirmed by monoclonal antibody based immunodot and *V.alginolyticus* at 96 h post infection. The shrimps in control group not fed either biofilm or free cells were positive to WSSV and *V.alginolyticus* up to the termination of the experiment and 120 h post infection, respectively. Histological lesions either in WSSV or *V.alginolyticus* infected shrimps were of grade C type in biofilm fed shrimps, grade B type in free cell fed shrimps and grade A type in control shrimps. The microscopic lesions of WSSV and *V.alginolyticus* infection were confirmed by immunoperoxidase and histological Gram's staining, respectively.

Conclusion

The results of the present study revealed that the biofilm cells of *Vibrio alginolyticus* can be used in *Penaeus monodon* to activate the immune system of the shrimps for protection against pathogens. Biofilm cells of *V.alginolyticus* were found to be better than free cells in actvating the immune system of shrimps. In the present study, increased phenoloxidase activity, total haemocyte count, total haemolymph protein and antibacterial activity were observed in biofilm fed shrimps and gave better protection against White Spot Syndrome Virus and *Vibrio alginolyticus* infection.

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7. REFERENCES

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APPENDIX

APPENDIX

1. Marine Anticoagulant Solution

Trisodium citrate	0.01 M
Sodium chloride	0.34 M
EDTA	10 mM
Glucose	0.12 M

2. Phosphate Buffer Saline

Sodium chloride	137 mM
Potassium chloride	2.7 mM
Sodium phosphate dibasic	1.4 mM
Potassium phosphate monobasic	4.3 mM
pH	7.4

3. Reducing Buffer

Tris HCl	0.5 mM
SDS	3per cent (W/V)
2-6, mercapitoethanol	5per cent (V/V)
Glycerol	10per cent (V/V)
Bromophenolblue	0.05per cent (W/V)

4. Cacodylate-citrate buffer

Sodium cacodylate	0·01 M
Sodium chloride	0·45 M
Trisodium citrate	0·10 M
pH	7·0

5. Cacodylate buffer

Sodium cacodylate	0·01 M
Sodium chloride	0.45 M
Calcium chloride	0.01 M
Magnesium chloride	0.26 M
pH	7.0

6. TNE buffer

Tris Hydrochloride	50 mM
Sodium chloride	400 mM
EDTA	5 m
pH	8.5

7. TM buffer

Tris hydrochloride	50 mM
Magnesium chloride	10 mM
pH	7.5

8. Neutral Buffered Formlin (10 per cent)

Sodium Phosphate monobasic	4 g
Sodium Phosphate dibasic	6 g
Formalin	100 ml
Distilled water	900 ml

9. Eosin (1per cent)

Eosin	1 g
Distilled water	100 ml